Comparative Toxicokinetics of TetraChloroBenzylToluenes (TCBTs) and PolyChloroBiphenyls (PCBs)

(met een samenvatting in het Nederlands)

Proefschrift

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LIST OF ABBREVIATIONS

AUC	Area under the concentration-time curve
BA	Bioavailability
CYP	Cytochrome P450
ECD	Electron capture detection
EROD	Ethoxyresorufin-O-dealkylase
G.I-tract	Gastro-intestinal tract
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
HRGC	High resolution gaschromatography
IV	Intravenous
LDL	Low density lipoprotein
Log K_{ow}	Logarithm of the octanol to water partition coefficient
MROD	Methoxyresorufin-O-dealkylase
OHT	Hydroxytestosterone
PB-PK	Physiologically based pharmacokinetic
PC	Partition coefficient
PCB	Polychlorinated biphenyl
PROD	Pentoxyresorufin-O-dealkylase
TCBT	Tetrachlorobenzyltoluene
TCDD	Tetrachlorodibenzo-p-dioxin
VLDL	Very low density lipoprotein

CONTENTS

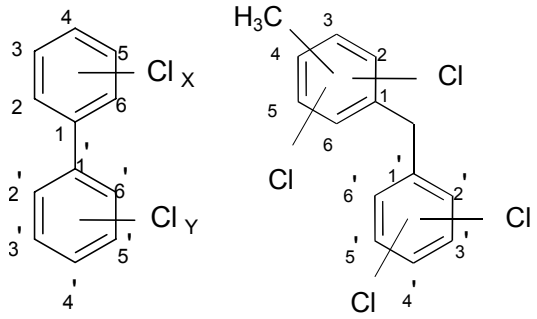
... Op basis van een deelverzameling van metingen kun je een logische consistente geschiedenis definiëren, waarvan je echter niet kunt zeggen dat die waar is; ze kan staande gehouden worden zonder tegenstrijdigheden…

(Elementaire deeltjes, M. Houellebecq)

CHAPTER 1

INTRODUCTION

Technical mixtures of polychlorinated biphenyls (PCBs) were widely used industrial chemicals due to their chemical stability, their thermal conductivity, their miscibility with organic solvents (*i.e.* lipophilicity), and their dielectric properties (Fig. 1A). By leakage from industrial devices, careless disposal practices, and accidents, PCBs were introduced into the environment where they persisted due to their chemical stability and their lipophilicity.



A B

Figure 1. The general chemical structure of a polychlorinated biphenyl (A) and tetrachlorobenzyltoluene (B). The 4 or 4′ positions are also known as *para*, the 3, 3′, 5, and 5′ positions as *meta* and the 2, 2′, 6, or 6′ positions as *ortho.*

These characteristics cause that PCBs accumulate in higher trophic levels of the food chain (Safe, 1994) and induce toxic effects like, acute lethality, hepatomegaly, fatty liver, porphyria, body weight loss, dermal toxicity, thymic atrophy, immunosuppressive effects, reproductive and developmental toxicity (Safe, 1994). Hence, international regulations restricted the use of PCBs during the 1970s. These regulations induced the development of alternatives for PCBs. Ugilec 141 (Fig. 1B) is one of these PCB substitutes. This chemical mixture, consisting of 69 different isomers (Ehmann and Ballschmiter, 1989), shares similar physicochemical properties with PCBs, like the lipophilicity (K_{ow}) and the water solubility (VanHaelst, 1996); Leonards and DeVoogt, 1989).

Ugilec 141 has been used as a hydraulic fluid in the mining industry in Germany since the early 1980s at approximately 1500 tons a year (Poppe *et al.*, 1988). Leakage from these hydraulic devices introduced TCBTs into the environment. In the rivers near and down stream the mines, levels of TCBTs between 0.1 and 25 mg. kg^{-1} tissue were found in fish (Friege *et al.*, 1989; Fuerst *et al.*, 1987a; Poppe *et al.*, 1988; Roennefahrt, 1987; Wammes *et al.*, 1997), which were comparable to PCB levels (Leonards and DeVoogt, 1989; Wammes *et al.*, 1997). Thus, TCBTs accumulated in fish like PCBs. Additionally, in mice Ugilec 141 appeared to induce biochemical changes similar to those induced by Aroclor 1254 but less pronounced. These changes included induction of cytochrome P450 1A1 enzyme activity and a decrease of plasma thyroxine levels (Murk *et al.*, 1991). In the rat the total amount of cytochrome P450 and CYP2B1 activity were marginally induced (VonMeyerinck *et al.*, 1990). Only a few studies have been performed to determine the tissue distribution and accumulation of Ugilec 141 in rats and in fish. Compared to PCBs the elimination of Ugilec 141 was faster and consequently, the accumulation was less pronounced for TCBTs (Bouraly and Millischer, 1989; VanHaelst *et al.*, 1996a; VanHaelst *et al.*, 1996b). Due to similarities with PCBs with respect to their chemical characteristics and environmental behaviour, the use of Ugilec 141 in new machinery was prohibited in the European Union in 1994. However, Ugilec 141 can still leak into the environment from old devices and pose possible health risks.

The most toxic PCBs induce their dioxin-like toxicity via interaction with the Ah-receptor (Safe, 1994). They have a planar conformation, which is an important requirement to interact with the receptor. One study has been performed to estimate, which TCBTs could adopt a coplanar conformation and could possibly interact with the Ah-receptor. Semi-empirical molecular modelling calculations of ring rotational

energy barriers were performed on the possible TCBT isomers (VanHaelst *et al.*, 1997). Comparing the energy barriers with PCBs resulted in only two (3,3'4,4'-Cl_4-2methyl- and 3,3',4,4'-Cl_4-5-methyl-chlorobenzyltoluene) TCBTs that could interact with the receptor and would induce dioxin-like toxicity.

KINETICS

The severity of toxic effects induced by xenobiotics depends on the concentration of the chemical at the site where the toxic effect occurs, *i.e.* the target site. Thus, insight in the relation between this internal concentration and the effect is required to determine at which concentration no detrimental effects occur. However, the concentration at the target site is the resultant of many factors like dose, physicochemical properties of the chemical and kinetics, *i.e.* processes that include absorption, distribution, metabolism and elimination.

Absorption and distribution

Absorption processes include uptake from the gastro-intestinal tract (G.I-tract) after oral ingestion, dermal uptake by exposure via the skin and uptake from the lung by inhalation. After absorption, the xenobiotics are transported by blood to remote tissue and organs, where they could deposit. This depends on the intrinsic properties of the tissues and the compound specific characteristics. Many xenobiotics that are absorbed from the GI-tract are transported via the portal vein to the liver where they can be metabolised before reaching the systemic circulation. This is called first-pass elimination. Numerous lipophilic substances, such as DDT and lipophilic vitamins, are transported via the lymph (Charman *et al.*, 1997) to the thoracic duct where the lymph mixes with the venous blood. Via this route of uptake the liver as first-pass eliminating organ is avoided, which entails a higher systemic concentration, *i.e.* a higher bioavailability (Kwan, 1997) relative to the portal vein uptake route.

Metabolism

Metabolism of xenobiotics is generally divided in two phases: phase I and phase II metabolism. During phase I metabolism xenobiotics are converted into more polar metabolites that can be readily excreted, or be conjugated during phase II metabolism. The conjugation is followed by biliary or renal excretion. The major enzyme system that is involved in phase I metabolism is Cytochrome P450 (CYP). This enzyme system is present in hepatic as well as in extrahepatic tissues (Gonzalez, 1992). The liver contains of the highest CYP concentrations followed by the

intestines and the lungs (De Waziers *et al.*, 1990). Within the cells, CYPs are primarily located in the endoplasmic reticulum and consist of many different isoforms with different substrate selectivity (Gonzalez, 1992). In addition, many of these enzymes are differentially inducible *e.g.* phenobarbital induces CYP2B enzymes while β-naphthoflavone induces CYP1A enzymes (Parkinson, 1996). The expression of CYP enzymes depends on species, dietary and genetic factors (Clarke, 1998). For example in fish, the CYP concentrations in the liver are much lower than in mammalian species (Sijm and Opperhuizen, 1989). Within the different species, CYP expression depends on the diet and the genetic constitution, which is responsible for inter-individual dissimilarities in enzyme activity and in metabolic rates between individuals of one species.

Elimination

Elimination is the process of excretion via urine, bile, feces, exhalation via the lungs, transpiration via the skin, and via lactation. In general metabolites are excreted via these routes, however, depending on the xenobiotic, excretion of parent compound also occurs. Small hydrofilic molecules could be eliminated as parent compound via the urine, and larger molecules can be excreted into the feces via the bile. Some metabolites like glucuronides can undergo metabolism in the gut by microflora, which might result in the reformation of the parent compound. Subsequently, this compound could be taken up again and metabolised in the liver. This process is called enterohepatic circulation. Enterohepatic circulation increases chemical half-lives, *i.e.* the time necessary to reduce the blood concentration with 50 %.

KINETICS OF PCBS

The kinetics of lipophilic substances like PCBs have been studied to large extent as reviewed by Matthews and Dedrick, (1984) and VandenBerg *et al.* (1994). One of the main characteristics with respect to the toxicokinetics of the polyhalogenated hydrocarbons is the slow elimination from the organism, resulting in life-time exposure to these chlorinated highly lipophilic compounds. During this exposition the organisms are prone to possible toxic effects, *e.g.* developmental and reproductive effects (Safe, 1994).

The fact that PCBs , almost 30 years after the ban on their use, are still being detected in biota, including humans (Dewailly *et al.*, 1999; Patterson *et al.*, 1994),

clearly illustrates the extended exposure periods. TCBTs are physicochemically related to PCBs, with respect to octanol to water partitioning (Log K_{ow} of tetra or higher chlorinated PCBs range from 5 to 7 and for the TCBTs from 6.5 to 7.5), conformational aspects, and dimensions. Hence, similarities between TCBTs and PCBs regarding their toxicokinetics can be anticipated, including a slow elimination of TCBTs from the organism and accumulation in the adipose tissue. As a consequence, TCBTs may pose similar risks as PCBs or other related polyhalogenated hydrocarbons, with respect to the possible accumulation of these compounds in the environment and biota. A short overview of kinetics of PCBs is presented here.

Absorption

Humans are generally exposed to PCBs via ingestion of contaminated food. The absorption efficiency from the G.I-tract appears to be inversely related to the degree of chlorination. In the rat the absorption efficiency ranges from 66 to 96% (Tanabe *et al.*, 1981; Fries *et al.*, 1985) while for human adults it ranges between 27 and 88 % (Schlummer *et al.*, 1998). Nursing infants absorb PCBs for more than 90 % (Dahl *et al.*, 1995; Korner *et al.*, 1992). The absorption efficiency also depends on the PCB concentrations in the blood and the gut tissue. High blood levels reduce the uptake which is assumed to be caused by reduced diffusion rates (Rohde *et al.*, 1999; Schlummer *et al.*, 1998).

After absorption from the G.I-tract, most of the PCBs enter the intestinal lymphatic drainage sequestered in chylomicrons, and are subsequently transported to the thoracic duct where the lymph mixes with the venous blood, as was demonstrated in dog and sheep (Busbee *et al.*, 1985; Ziprin *et al.*, 1980). In addition, it was shown that di-chlorinated PCBs were taken up via the portal vein (Busbee and Ziprin, 1994).

Once PCBs have entered the bloodstream, they equilibrate rapidly over lipoproteins and albumin in the plasma (Becker and Gamble, 1982; Borlakoglu *et al.*, 1990c; Busbee *et al.*, 1985; Maliwal and Guthrie, 1982; Matthews *et al.*, 1984; Mohammed *et al.*, 1990). These studies suggested that the PCBs are transported to remote tissue and organ via non-covalent binding to lipoproteins and albumin.

In vitro it was demonstrated that lipoproteins and albumin facilitated the uptake and the elimination of the PCBs. In addition, the tissue disposition of PCBs depended also on the lipid solubility of the congeners (Di Francesco and Bickel,

1985; Di Francesco *et al.*, 1988; Guo *et al.*, 1987; Rau and Vodicnik, 1986; Shireman, 1988).

Distribution and disposition

PCBs in the bloodstream are initially distributed to the liver and the muscle, which can easily be explained by the fact that the liver is a well-perfused organ and the muscle is about the largest tissue in volume relative to the total body composition. After this initial uptake the PCBs slowly redistribute to skin and adipose tissue because of the high lipid content of these tissues (Matthews and Dedrick, 1984; Matthews and Anderson, 1975) and the relatively low perfusion rates of these tissues. As a consequence, the adipose tissue and skin reach their maximum levels at later time points than *e.g.* the liver. Many studies have shown that among different mammalian species, like rat, mice, dog, and monkey, the major storage tissue for PCB congeners is adipose tissue, followed by skin, muscle, liver and blood (Heinrich Hirsch *et al.*, 1997; Lutz *et al.*, 1977; Lutz *et al.*, 1984; Matthews and Dedrick, 1984; Moir *et al.*, 1996; Morales *et al.*, 1979; Sipes *et al.*, 1982a; Sipes *et al.*, 1982b).

Congener specific distribution was observed (Lutz *et al.* 1977; Matthews and Anderson, 1975; Nims *et al.*, 1994; Morales *et al.* 1979), which depended on the degree of chlorination, the substitution pattern (Borlakoglu *et al.*, 1991; Nims *et al.*, 1994), and the tissue (Nims *et al.*, 1994). Some congeners like 3,3',4,4'5-PnCB are specifically retained by the liver, due to binding to cytochrome P450 1A2 enzyme (Andersen *et al.*, 1997; Andersen *et al.*, 1993; DeVito *et al.*, 1998; VanBirgelen *et al.*, 1994a; Voorman and Aust, 1987; Yoshimura *et al.*, 1984), resulting in liver to blood partition coefficients that are higher than expected based on the solubility of the PCB in the lipid fraction of both fractions.

Physiology *e.g.* tissue volumes and perfusion rates, also plays a role in tissue distribution, (Birnbaum, 1983; Matthews and Tuey, 1980; Wyss *et al.*, 1982; Wyss *et al.*, 1986). As PCBs accumulate in adipose tissue, the volume of the adipose tissue affects the tissue distribution of these congeners. A larger volume implies that a larger amount of the PCBs distribute to the adipose tissue (Birnbaum, 1983). This entails that the other organs or tissues receive a smaller fraction of the body burden. Compared to rats humans have larger fat depots. Thus, the tissue distribution in humans is expected to be different from rats.

Metabolism

Metabolism of PCBs occurs mainly in the liver (Lutz *et al*., 1977; Matthews and Dedrick, 1984) and is achieved primarily by cytochrome P450 (CYP) enzymes (Matthews and Dedrick, 1984) by hydroxylating the PCBs. This is considered the rate limiting step in the elimination of PCBs (Lutz *et al*., 1977; Matthews and Dedrick, 1984). Thus, after the initial hydroxylation the compound can be readily excreted or further metabolised. Further metabolism includes additional hydroxylation reactions or conjugation reactions with *e.g.* glutathione or glucuronic acid. These metabolites are excreted by urine or via the bile, into the feces. Some general conclusions, with respect to metabolism of PCBs, have been drawn (Matthews and Dedrick, 1984; Safe, 1989):

- Hydroxylation reactions are preferably directed to the *para* position in the least chlorinated phenyl-ring, except when this site is sterically hindered,
- in lower chlorinated biphenyls the *para* positions of both rings and the carbon atoms that are *para* to the chlorine atoms are easily metabolised,
- the presence of vicinal unsubstituted carbon atoms facilitates hydroxylation of PCBs,
- the degree of chlorination on both rings is inversely related to the metabolic rate,
- metabolism of individual PCBs is species dependent.

In vitro studies using purified CYP enzymes showed that CYP2B enzymes catalysed the hydroxylation of *ortho* substituted PCBs while CYP1A enzymes catalysed preferably the hydroxylation of non-*ortho* substituted PCBs (Kaminsky *et al*., 1981). CYP2B1 preferably hydroxylated PCBs at the unchlorinated phenyl-ring while CYP1A1 showed an inclination towards the chlorinated phenyl ring (Kaminsky *et al.,* 1981). These results demonstrate that the hydroxylation of PCBs by CYPs is highly regio-selective and depends on the enzyme that catalyses the reaction. Thus, the metabolic rates of PCBs depend on the CYP composition in the liver and extrahepatic tissue as well as on the congener itself.

Metabolism of PCBs in extrahepatic tissue has not been reported. However, the presence of various CYP enzymes in extrahepatic tissue like small intestine, kidney and lung suggest possible Phase I metabolism. Within the G.I-tract it has been reported that microflora can deconjugate conjugated PCBs (Bergman *et al*., 1982; Gustafsson *et al*., 1981), but hydroxylation of PCBs by microflora in the G.I-tract has not been reported.

Elimination of metabolites

The urine, bile and feces are the main routes of excretion of metabolised PCBs. The contribution of urinary excretion to the total excretion of PCBs decreases with increasing number of chlorine atoms in mammals, in favour of excretion via the feces (Fisher *et al.*, 1989; Lutz *et al.*, 1977; Lutz *et al.*, 1984; Matthews and Anderson, 1975; Matthews and Tuey, 1980; Morales *et al.*, 1979).

Elimination of parent compound

PCBs are mainly excreted as metabolites but elimination of parent compound occurs as well. For the hardly metabolisable 2,2',4,4',5,5'-HxCB only 2 % of the dose was excreted in the feces as parent compound after 21 days and about 2 % as metabolite (Birnbaum, 1983). A different study with this PCB showed that the percentage of the dose excreted in the feces increased up to 12 % forty weeks after exposure (Muhlebach and Bickel, 1981). For slowly metabolisable PCBs the contribution of excretion of parent compound to the overall excretion is relatively high. Less then 10% of the dose of the readily metabolisable 2,2', 3, 3',6, 6'-HxCB is excreted as parent compound four days after exposure in the feces (Birnbaum, 1983). These results imply that excretion of parent compound contributes marginally to the total elimination of easily metabolisable PCBs. Taking into account the lifetime exposure of humans to PCBs with half-lives between 2 to 6 years, the excretion of parent compounds for the slowly metabolised PCBs might become relevant. A less continuous route of excretion is via lactation. Its contribution to the total excretion however, could be approximately 96 % of the total body burden during one lactation period, dependent on the species (Takagi *et al.*, 1976; Vodicnik, 1986; Gallenberg *et al.*, 1990; Gallenberg and Vodicnik, 1989).

Pharmacokinetic modelling

Kinetic processes can be described quantitatively using mathematical descriptions in pharmacokinetic models. Two main types of pharmacokinetic models can be distinguished: data based pharmacokinetic models and physiologically based pharmacokinetic (PB-PK) models. For a comprehensive review the reader is referred to the papers of Yang and Andersen (1994) and Krishnan and Andersen (1994). Data-based pharmacokinetic models represent the organism as one or more compartments. The parameters of the model are obtained by fitting the model to the kinetic data, and have no direct physiological or biological meaning. As a consequence, the parameter values are of limited value with respect to their biological meaning. They describe tissue or blood concentration of one particular experiment

and can not be used for extrapolation to other experimental conditions. The physiologically based models represent the organism as compartments that have a physiological meaning and are based on an anatomical description like tissue volume and blood flow. Furthermore, the model description includes biochemical processes like metabolism. These PB-PK models comprise many parameters. A large number of them can be obtained *a priori* from the literature like *e.g.* blood flows, cardiac output and tissue volumes, or can be determined experimentally or *e.g.* from *in vitro* experiments. The advantages of these physiologically based models are that they can forecast tissue concentrations under circumstances that have not been experimentally determined, *e.g.* different dose levels, different routes of exposure, or different exposure regimen. In addition, these models can also be used to extrapolate the kinetics to other species of species by scaling tissue volumes and blood flows, *e.g.* from rat to human (Krishnan and Andersen, 1994). Using this type of modelling the estimation of the tissue concentration is improved or at least the underlying assumptions are indicated.

Parameterisation

The partition coefficients (PC) quantify the distribution of chemicals between tissues and blood under steady state conditions. Great effort has been put into the development of methods to estimate or determine PCs on forehand for use in PB-PK models. Different model approaches have been proposed to estimate PCs. These model approaches are based on distribution of a chemical between the lipids and the aqueous phase of a tissue and blood. This distribution is directed by the n-octanol to water partition coefficient (=K_{ow}) (DeJongh *et al.*, 1997; Haddad *et al.*, 2000; Poulin and Theil, 2000). These models were validated for compounds, having a Log K_{ow} between 2 to 5.

Furthermore, *in vitro* methods were developed to determine PCs experimentally (Artola-Garicano *et al.*, 2000; Jepson *et al.*, 1994; Murphy *et al.*, 1995). The *in vitro* methods for non-volatile chemicals measure the partitioning between tissue and saline or blood and saline. These PCs agreed well with values determined *in vivo* (Murphy *et al.*, 1995).

Many *in vivo* and *in vitro* methods have been described to determine metabolic parameters. For volatile chemicals independent metabolic parameters were obtained using closed chamber experiments. The change of chemical concentration in the chamber, was used to determine the uptake and the metabolism by the animal

(Andersen *et al.*, 1980; Gargas *et al.*, 1986). This procedure was suitable to obtain metabolic rate constants for volatile chemicals, which could be described by Michaelis-Menten kinetics.

Another way to obtain independent metabolic parameter values is by *in vitro* studies. Subcellular fractions like microsomes, isolated cells, tissue slices or isolated perfused organs are used to determine metabolic rates. These systems differ in their degree of tissue or organ integrity. Therefore, it remains difficult to determine the relevance of the *in vitro* metabolic rates for the *in vivo* situation, *e.g.* scaling of metabolic rate constants in hepatic microsomal fractions that contain enzymes that are located in the endoplasmic reticulum to a whole liver. This scaling involves many uncertainties. For example the extrapolation of transport processes in the liver and in the *in vitro* system or in binding characteristics *in vivo* compared to *in vitro*. However, some studies showed the validity of *in vitro* metabolic rate constants to predict *in vivo* metabolic rates, using hepatocytes, liver slices or microsomal fractions (Carlile *et al.*, 1998; Hayes *et al.*, 1995; Houston, 1994a; Houston, 1994b; Obach, 1997; Worboys *et al.*, 1996; Worboys *et al.*, 1997).

Absorption rate constants and excretion rate constants can be determined *in vivo* and *in vitro*. *In vivo* absorption or excretion rates are determined by fitting a mathematical description to the experimental data, which are in general tissue or blood concentration data. *In vitro* experiments involve isolated tissue preparation like for example skin slices. For PCBs a few *in vitro* studies have been performed to determine dermal absorption rates (Fisher *et al.*, 1989; Wester *et al.*, 1983). For intestinal absorption also *in vitro* studies have been done (Dulfer *et al.*, 1996; Oomen, 2000) using Caco2-cells.

SCOPE OF THIS THESIS

TCBTs were produced to replace PCBs because congeners of the latter group have been accumulating in biota and have been inducing toxic effects. Accumulation of PCBs is partly caused by their resistance towards metabolism. To study the possible accumulation of TCBTs in biota, the rate of metabolism can be used to estimate their potential to accumulate in biota. CYP enzymes catalyse the hydroxylation reaction of PCBs, which is assumed to be rate limiting with respect to the elimination of these compounds. Based on the chemical structure of the TCBTs it is fair to assume that CYP enzymes also hydroxylate TCBTs, and that this is rate

limiting the elimination as well. Hence, metabolic rate constants of TCBTs were determined *in vitro* and compared to metabolic rates of PCBs. The TCBTs that could adopt a coplanar conformation (3, 3', 4, 4'-Cl_4-2-methyl- and 3, 3', 4, 4'-Cl_4-5-methyl-chlorobenzyltoluene, referred to as TCBT 87 and TCBT 88, respectively) were examined in these experiments because of their supposed interaction with the Ah-receptor, which is related to dioxin-like toxicity. TCBT 94 (3, 3', 4', 5-Cl_4-4-methyl-chlorobenzyltoluene) was included as well, representing differently substituted TCBTs. Chapter 2 describes an *in vitro* method to determine metabolic rate constants of TCBTs using rat and fish hepatic microsomes. This method was also used in chapter 3 and 4. In these chapters metabolic rates of TCBTs were compared with tetra, penta and hexa chlorinated PCBs using microsomes from rat (Chapter 3) and humans (Chapter 4). Many tissues express various CYPs that could be involved in the metabolism of the TCBTs or PCBs. Thus, identifying the CYPs provides insight in the extrahepatic tissues contributing to their metabolism. Chapter 3 describes the identification of CYPs metabolising the TCBTs and the PCBs in the rat by correlation analysis. Rats were pretreated with different inducing agents to increase the activity of specific CYPs. Statistical analysis of the correlations between specific enzyme activities and metabolic rate constants of the TCBTs and the PCBs revealed the possible involvement of a specific CYP. Subsequent selective inhibition of correlating CYP confirmed the involvement of this enzyme. In chapter 4 the identity of the CYPs in humans were also determined by correlation analysis using human liver microsomes.

An additional goal of this study is to gain more insight in the kinetics of three TCBTs using PB-PK modelling. Chapter 5 describes the *in vivo* kinetics of these TCBTs after a single intravenous bolus injection. The tissue concentration data were used to calibrate the PB-PK model. To determine the value of the *in vitro* metabolic rate constants from hepatic microsomes for the parameterisation of PB-PK models, the *in vitro* metabolic rate constants from chapter 2 were compared to the *in vivo* values. In general, the exposure of mammals to these xenobiotics occurs mainly via oral ingestion. This exposure route might influence the bioavailability by reduced absorption or by first-pass metabolism. In chapter 6 the oral bioavailability of some TCBTs and some PCBs have been determined. Rats were exposed to a single intravenous injection or a single oral dose of a mixture of PCBs and TCBTs. Tissue concentrations were determined and analysed by a PB-PK model for the TCBTs and for the PCBs. The studies described in chapter 5 and 6 provide insight in the *in vivo*

behaviour of highly chlorinated lipophilic substances in the rat. The PB-PK model for the PCBs and the TCBTs in the rat was extrapolated to humans to estimate the tissue concentrations for different exposure regimen (Chapter 7). The tissue volumes and the blood flows in the rat were adjusted to the human values. The metabolic rate constants from the human liver microsomes were extrapolated to *in vivo* metabolic rate constants taking into account the microsomal volume in the human liver. With this model, tissue levels of TCBTs in humans after a single or multiple doses were estimated (Chapter 7) and compared to PCB levels observed in human tissues.

CHAPTER 2

BIOTRANSFORMATION RATES OF UGILEC141® (TETRACHLOROBENZYLTOLUENES) IN RAT AND TROUT MICROSOMES

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ABSTRACT

In vitro biotransformation rate constants of tetrachlorobenzyltoluene (TCBT) isomers 3,3',4,4'-Cl_4-2-Me (TCBT 87), 3,3',4,4'-Cl_4-5-Me (TCBT 88), and 3,3',4',5-Cl_4-4-Me (TCBT 94) were determined using trout and rat hepatic microsomes. The disappearance of the TCBTs from the *in vitro* system followed first-order kinetics. The estimated biotransformation constants (*k*) for the rat ranged from 0.96 to 4.14 h^{-1}. Biotransformation rates for trout microsomes were much lower and ranged from 0.009 to 0.017 h^{-1}.

INTRODUCTION

Ugilec 141®, an industrial mixture of 69 tetrachlorobenzyltoluene (TCBT) isomers (Ehmann and Ballschmiter, 1989), was introduced as a replacement for polychlorinated biphenyls (PCBs) on the European market in the early 1980s. The use of PCBs was prohibited because of their environmental persistence and their toxicity. Due to structural and physicochemical similarities with PCBs, food chain accumulation of TCBTs was expected (VanHaelst, 1996) and therefore its use was prohibited in the European Union. In fish levels up to 24.9 $mg.kg^{-1}$ fish tissue have been reported (Fuerst *et al.*, 1987b). Weak accumulation of Ugilec 141® has been demonstrated in fish and rat (Bouraly, 1989). Bioconcentration factors (BCFs) for Ugilec 141® in rat liver are lower than for Fenclor 54®, a PCB mixture (Leoni *et al.*, 1988). BCFs in the guppy (*Poecilia reticulata*) were lower for six TCBTs than for 2, 2', 4, 4', 5, 5'-hexachlorobiphenyl (VanHaelst *et al.*, 1996a). Ugilec 141®, Aroclor 1254® and 3, 3', 4, 4'-tetrachlorobiphenyl (PCB 77), induced comparable biochemical changes such as enzyme induction in both mice and rats, although the effects were less pronounced for Ugilec 141® (Murk *et al.*, 1991; VonMeyerinck *et al.*, 1990). It was hypothesized that the limited accumulation as well as the limited cytochrome P450 (CYP) induction is explained by a relatively rapid biotransformation of TCBTs (Murk *et al.*, 1991; VonMeyerinck *et al.*, 1990).

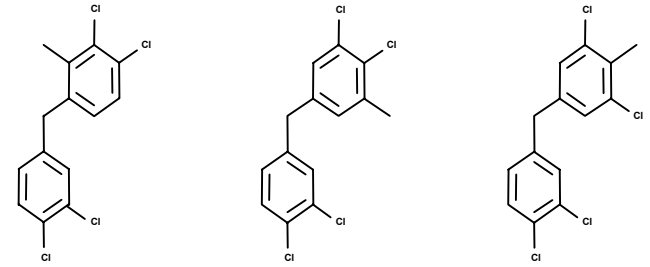


Figure 1. Chemical structure of three TCBT isomers.

The relationship between biotransformation and accumulation has been well known for PCBs (Muehlebach *et al.*, 1991). In contrast, little is known about biotransformation of TCBTs. In order to explain the limited accumulation in greater

detail, we determined biotransformation rate constants of three TCBT isomers (Fig. 1) in rat and trout microsomes as representatives of mammalian and aquatic species.

The theoretical affinity of all TCBTs for the Ah-receptor was previously estimated by computer simulation (VanHaelst *et al.*, 1997). TCBTs 87 and 88 showed a high probability to fit into the Ah-receptor and may therefore exhibit some forms of dioxin-like toxicity, which can be relevant for risk assessment.

MATERIALS AND METHODS

Chemicals

The 3,3',4,4'-Cl_4-2-Me- (TCBT 87), 3,3'4,4'-Cl_4-5-Me- (TCBT 88), and 3,3',4',5-Cl_4-4-Me- (TCBT 94) tetrachlorobenzyltoluene (purity >99%) were synthesised as described previously (DeLang *et al.*, 1998). The 4,4'-dichlorobiphenyl (PCB 15) and 2,2',4,4',5,5'-Hexachlorobiphenyl (PCB 153) (purity >99%) were obtained from Dr Ehrenstorfer GmbH (Ausburg, Germany). NADP (β-NADP), glucose-6-phosphate-dehydrogenase (G6PDH) (140 U/mg), and glucose-6-phosphate (G6P) were purchased from Boehringer-Mannheim (Almere, The Netherlands). n-Hexane, methanol, acetone, and glycerol (analytical grade) were obtained from J.T. Baker B.V. (Deventer, The Netherlands). The incubation buffer (KPi, pH=7.4) consisted of 0.08 M K_2HPO_4, 0.01 M Na-EDTA, and 0.08 M KH_2PO_4 (Merck, Amsterdam, The Netherlands). Resorufin, ethoxyresorufin, pentoxyresorufin, methoxyresorufin and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, USA).

Animals

Male Wistar CPB:uWU rats (250-300 g) from Central Animal Laboratory (Utrecht University) remained untreated or were given a single intraperitoneal dose of either β-naphtoflavone (BNF; 67 mg/kg in arachid oil) or phenobarbital (PB; 64 mg/kg in 0.9 % saline). The livers were removed 24 hours later under anaesthesia (Nembutal®, 200 mg/kg) after *in situ* perfusion of the liver via the portal vein with 0.9 % saline. Rainbow trout (*Oncorhynchus mykiss*) (300-400 g), purchased from a local hatchery, were reared at 15°C. Trout were killed and the livers were quickly removed and weighed.

Preparation of microsomes

Isolation of microsomes and determination of protein content was performed as described previously (Lowry *et al.*, 1951; Rutten *et al.*, 1987).

Biotransformation assay

The present method was based on a previously described, qualitative biotransformation assay (Boon *et al.*, 1998). All substrates were dissolved in acetone. The final solvent concentration was limited to 1.6 % to prevent influence on the microsomal enzyme activity (Cotreaubibbo *et al.*, 1996). The three TCBT substrates were incubated in glass test tubes in a gently shaking water bath at 37°C (rat) or 15°C (trout). The incubation mixture (final volume 1220 µl) consisted of KPi buffer, a regenerating system (2.5 mM $MgCl_2$, 8 mM G6P, 4.3 U G6PDH), TCBT substrate (final concentrations 5.1, 26, 51 or 290 nM), and 1 mg microsomal protein. The TCBTs were incubated individually with PCB 153 and 15 as a negative and positive biotransformation control, respectively. A mixture of TCBTs 87, 94 and both PCBs, which were all present at a concentration of 290 nM was incubated with rat microsomes to detect possible substrate competition. The biotransformation reaction was started by adding 2 mM NADP (final concentration). Instead of NADP, 100 µl KPi was added to obtain data for non-enzymatic disappearance of TCBT. The biotransformation reaction was stopped at 0, 5, 15, 30, 40, 60 or 90 minutes (rat) and at 0, 4, 8 and 11 hours (trout) by adding 1000 µl ice cold MeOH to the incubation mixture.

Extraction

Substrates were extracted from the incubation buffer by three n-hexane washings (2 ml) using a Vortex shaker (Boom B.V. Meppel, the Netherlands). The organic phase was collected after centrifugation (1000 g, 2 min). After evaporation (T = 20 °C) of the hexane, the samples were redissolved in 100 µl n-hexane. Recovery of PCB 153 and TCBTs from control incubations (without NADP) were always > 90%.

GC-analysis

Extracts were analysed using a Carlo-Erba Mega Series HR-gas chromatograph, equipped with a ^{63}Ni-Electron Capture Detector and a DB-5 column (15 m, dl=0.25 µm, ID=0.32 mm). Injector temperature: 225 °C; split ratio 30 (top split =60 ml/min and bottom split: 2 ml/min) injection volume: 2 µl; temperature program: 1 min at 225 °C; 5 min at 250 °C, rate 30°C/min. The detection limit was 20 pg.

The individual data were plotted using Slidewrite for Windows 4.0 (Advanced Graphics Software Inc., Carlsbad, USA) and fitted according to first-order kinetics:

$$A(t) = A_0 e^{-kt} \qquad \text{eq. 1}$$

where A is the amount at time t, A_0 is the amount at t=0 defined as 100%, and k (time^{-1}) is the biotransformation rate constant. Statistically significant differences between the estimates of the biotransformation rate constants were determined by Student t-test with Bonferroni adjustment (α=0.05/3).

Enzyme activity assays

Enzyme activity of control, BNF and PB-induced microsomes was measured using pentoxy-, ethoxy-, and methoxyresorfin (=PROD, EROD, MROD, respectively) as model substrates for CYP 2B and 1A activity (Burke *et al.*, 1985). Microsomes from untreated animals were incubated for 140 min at 37 °C (rat) or 12 hours at 15 °C (trout). Their activity was monitored by measuring EROD activity, as indicator for the possible loss of microsomal enzyme activity over time.

RESULTS

Biotransformation appeared to be linearly dependent on the substrate concentration (5-290 nM) for each TCBT isomer at concentrations near aqueous solubility (Fig. 2).

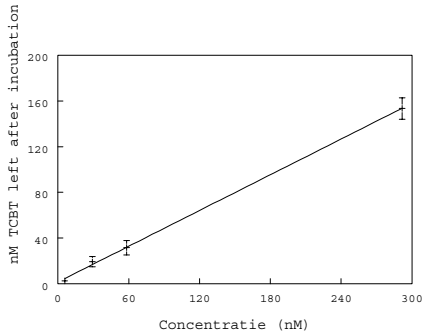
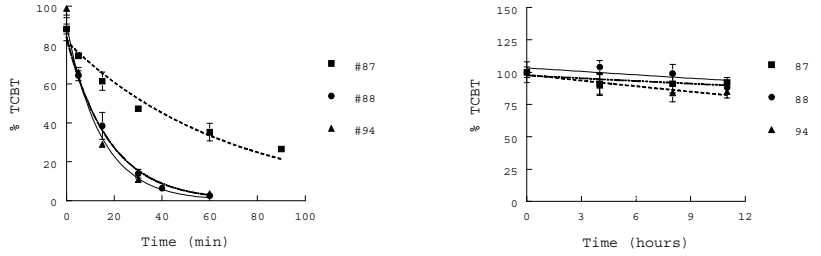


Figure 2. Concentration dependency of the biotransformation of TCBT 88 after 20 minutes of incubation using rat hepatic microsomes. Disappearance of parent compound (TCBT 88) from the incubation mixture was used as measure for biotransformation. Each data point is the average of three determinations with standard error.

Biotransformation rate constants (*k*) and half-lives were derived from plots showing the relative disappearance of each isomer (Table 1; Fig. 3). Biotransformation of the positive control (PCB 15) was only detected in control samples (no NADP added) and in samples from incubations with rat microsomes at t=0, while for trout microsomes 60% of PCB 15 was recovered after 4 hours of incubation. The average non-enzymatic disappearance of TCBT was 6.0 ± 2.1 % for both species. For the rat, significant differences in biotransformation rates were observed among the individual isomers (TCBT 94 > 88 >> 87).



A B

Figure 3. Disappearance curves of three TCBT isomers using rat (A) and trout (B) hepatic microsomes. Incubations (average ± SD, n=3) were performed at 37 °C (rat) or 15 °C (trout).

When a mixture of TCBT 87 and 94, PCB 15 and 153 was incubated, biotransformation rate constants were not significantly different from incubation mixtures with the single isomers (data not shown). Thus, no substrate competition occurred up to concentrations of 290 nM.

The data for trout do not show unambiguously that biotransformation of TCBT occurs. The *k*-values for trout microsomes are two to three orders of magnitude lower than for rat microsomes (Table 1). Incubating trout microsomes at higher temperatures (15, 22 and 26 °C) did not influence the biotransformation rates (data not shown). Due to extremely low biotransformation of TCBTs by trout hepatic microsomes within the observed 11 h incubation period, no significantly different biotransformation rates among isomers could be observed.

Table 1. First-order biotransformation rate constants *k* (95% confidence interval), and half-lives for three TCBTs for rat and rainbow trout.

TCBT	k (upper limit of 95% C.I.)		Half-life (min)	
	Rat (min^{-1})	Trout (h^{-1})	Rat (min)	Trout (min)
87	0.016* (0.021)	0.009 (0.015)	43	$4.8x10^3$
88	0.063 (0.074)	0.012 (0.015)	11	$3.5x10^3$
94	0.069 (0.096)	0.018 (0.017)	10	$2.4x10^3$

* TCBT 87 is statistically significantly different from TCBT 88 and 94 ($p<0.001$)

The present method led to accurate determination of *k*-values for biotransformation of all three isomers in the rat, as illustrated by the narrow 95% confidence intervals. For trout, where biotransformation was relatively slow, the method resulted in much larger confidence intervals.

Table 2. Influence of phenobarbital or β-naphtoflavone pretreatment on enzyme activities of rat hepatic microsomes ($pmol.min^{-1}.mg^{-1}$).

Treatment	EROD	MROD	PROD
Untreated	26.9	80.1	9.9
Phenobarbital	15.7	27.8	55.5
β-naphtoflavone	48.1	225.3	14.6

Pretreatment of rats with either PB or BNF led to induction of microsomal activity towards the model substrates for CYP1A1, 1A2 and 2B1 of up to five times (Table 2). PB induced microsomes metabolised the TCBTs 10 to 16 times more rapidly than the corresponding control microsomes, while BNF induced microsomes metabolised TCBTs only 1.5 to 1.7 times more rapidly (Fig. 4). Loss of biotransformation activity of microsomes overtime was reflected by a decrease of EROD activity (Fig. 5). For the rat, a gradual decrease to 75% of the initial activity was observed within 140 min. For trout, the activity decreased to 50% in 4 hours. Thereafter, the average activity remained constant.

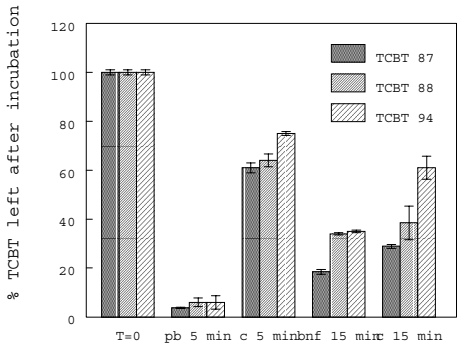


Figure 4. Effect on the disappearance of TCBT using hepatic microsomes from rats pretreated with PB and BNF compared to the disappearance of TCBT using hepatic microsomes from untreated rats (C). Each bar represents the mean (±SD) of three determinations.

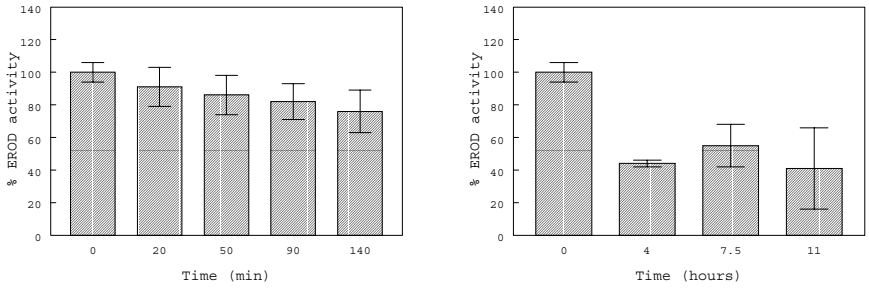


Figure 5. Relative decrease of EROD activity in rat (left panel) and trout (right panel) hepatic microsomes during the incubation period of the TCBT biotransformation assay. Each bar represents the mean (±SD) of three determinations.

DISCUSSION

Biotransformation rate constants of three TCBT isomers were obtained by measuring the disappearance of the parent compound from the *in vitro* system using microsomes from rat and rainbow trout. The non-enzymatic elimination attributed to only 6 % of the overall disappearance after 90 minutes in rat hepatic microsomes. Thus, disappearance of TCBT was almost entirely due to enzymatic biotransformation. The non-enzymatic elimination in trout was also 6 %, while the overall disappearance of TCBTs was maximally 10 % after 11 hours with relatively

high standard deviations. The *k*-values for trout did not differ significantly from zero and therefore the biotransformation rates can be considered semi-quantitative.

For PCBs, the metabolic rate is inversely correlated with the degree of chlorination (Borlakoglu and Wilkins, 1993; Grant *et al.*, 1972; Hutzinger *et al.*, 1972; Mehendale, 1976). Therefore we wanted to compare our *in vitro* biotransformation rates with metabolic rates for tetrachlorinated PCBs. However the availability of such data was limited. For 2,2',3,5,5',6-HCB (PCB 151), a Vmax of 0.45 pmol.min^{-1}.mg^{-1} microsomal protein was determined based on the formation of PCB 151 metabolites (Borlakoglu *et al.*, 1990a). BNF induced microsomes (123 fold EROD induction relative to control microsomes) metabolised 3,3',4,4'-TCB (PCB 77) at a Vmax of 240 pmol.min^{-1}.mg^{-1} (Morse *et al.*, 1995). Assuming linearity between the induction level and biotransformation rate, this implies a Vmax of 1.95 pmol.min^{-1}.mg^{-1}. However, this assumption may overestimate metabolism, as EROD activity does not necessarily reflect the specific enzymes that metabolise PCB 77. In the present study, the biotransformation rate was linear between 6-300 pmol parent compound per incubation (Fig. 2). For TCBT 88 (k = 0.063 min^{-1}.mg^{-1}), the biotransformation rate would be 18.9 pmol.min^{-1}.mg^{-1} microsomal protein at 300 pmol. The previous estimation suggests that the three TCBTs are metabolised more rapidly than either PCBs 77 or 151.

In the present study, large species differences were observed in the TCBT biotransformation rates between rat and trout microsomes (Table 1; Fig. 3). Metabolism of xenobiotics is generally higher in rats than in fish (Sijm and Opperhuizen, 1989). This is explained in part by a 30-60% higher total CYP concentration and a 3-36 % higher CYP activity per mg microsomal protein in mammalians than in fish (Sijm and Opperhuizen, 1989). However, these general observations do not explain the specific differences in TCBT metabolism between rat and trout. It was presently observed in rat microsomes (Fig. 4) that TCBTs are more efficiently metabolised by PB inducible CYPs (*e.g.* CYP2B) than by BNF inducible CYPs (*e.g.* CYP1A). This suggests that the limited biotransformation of the TCBT isomers in trout microsomes might be explained by the absence, or limited activity, of CYP2B enzymes in trout (Stegeman, 1989). Although an enzyme that immunologically cross-reacts with rat CYP2B proteins has been found in trout, it remains unclear whether this protein is functionally comparable to rat CYP2B enzymes (Stegeman, 1989).

For the structurally related PCB 77, it was recently demonstrated that CYP1A is involved in its metabolism in marine scup (*Stenotomus chrysops*). In contrast, CYP1A was identified in trout hepatic microsomes but PCB 77 was not metabolised measurably in this system (Murk *et al.*, 1994). This may explain the relatively high accumulation of PCB 77 in environmental fish tissue samples (Boer *et al.*, 1993). Thus, our present findings on the slow biotransformation rate of TCBTs in trout suggest a similar accumulation potential for these compounds in fish as for PCB 77.

In conclusion, the present study has reported approximately 100-fold differences in *in vitro* biotransformation rates of three TCBT isomers between rat and trout microsomes. These differences may result in a relatively high accumulation of TCBTs in trout. First-order biotransformation kinetics of these TCBTs were observed in the rat at concentrations near their aqueous solubility. The substantially lower biotransformation rate of TCBTs in trout compared to rat is probably explained by relatively lower or lack of CYP2B levels in trout, but this awaits further study.

CHAPTER 3

METABOLIC RATE CONSTANTS OF UGILEC 141 ISOMERS AND POLYCHLORINATED BIPHENYL CONGENERS USING RAT HEPATIC MICROSOMES AND THE IDENTIFICATION OF INVOLVED CYTOCHROME P450 ENZYMES

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ABSTRACT

Ugilec 141®, consisting of tetrachlorobenzyltoluenes (TCBTs) was introduced as a replacement for technical mixtures of polychlorinated biphenyls (PCBs). Accumulation of these TCBTs in biota occurred, indicating that their metabolism is limited. In the present study rat hepatic microsomes were used to determine metabolic rate constants for TCBTs and PCBs. Additionally, cytochrome P450 (CYP) enzymes involved in the metabolism of three TCBTs and eight PCBs were identified using correlation analysis. Rats were pretreated with specific cytochrome P450 inducers like: phenobarbital (CYP2B1/2), clofibric acid (CYP4A1), isosafrole (CYP1A2), β-naphthoflavone (CYP1A1), and dexamethasone (CYP3A1/2). CYP enzyme activities of the isolated microsomes as well as the metabolic rate constants of the TCBTs and the PCBs were determined and submitted to correlation analysis. Statistical significant correlations were confirmed using specific CYP inhibitors like orphenadrine (CYP2B1 inhibitor), Troleandomycin (CYP3A1 inhibitor) and α-Naphthoflavone (CYP1A2 inhibitor). It was shown that TCBTs were 2 to 50 times faster metabolised than PCBs. CYP 2B1 and CYP2B2 play a major role in the metabolism of the TCBTs. Involvement of CYP3A1, 3A2, 2A1 and 2A2 was indicated as well, but was less pronounced. Similar enzymes were involved in the metabolism of PCB 136. CYP1A1 and 1A2 played a major role in the metabolism of PCB 77. In conclusion, the more rapid metabolism of the TCBTs suggests that these compounds accumulate less than the PCBs.

INTRODUCTION

Ugilec 141®, an industrial mixture of 69 tetrachlorobenzyltoluenes (TCBTs) (Ehmann and Ballschmiter, 1989), was introduced as an alternative for polychlorinated biphenyls (PCBs) in hydraulic devices. Following the commercial introduction of TCBTs, tissue levels up to 25 mg. kg^{-1} fish were reported (Fuerst *et al.*, 1987b), indicating leakage of TCBTs into the environment and accumulation in the food chain, similar to PCBs. These factors resulted in 1994 in the prohibition on the use of TCBT in the European Union.

PCBs are ubiquitously present in the environment and accumulate in adipose tissue of species at different levels of the food chain. This bioaccumulation can be explained by slow elimination caused by slow biotransformation of PCBs. Biotransformation of PCBs is mainly performed in the liver (Lutz *et al.*, 1977; Matthews and Dedrick, 1984). The rate limiting step in the metabolism of PCBs is assumed to be the hydroxylation or oxidation by cytochrome P450 (CYP) enzymes (Lutz *et al.*, 1977; Matthews and Dedrick, 1984). TCBTs were shown to be metabolised by these enzymes as well (Kramer *et al.*, 2000b).

The liver contains many CYPs in various amounts. Insight in the identity of CYPs, involved in the metabolism of PCBs and TCBTs, would improve insight in the toxicokinetics of these compounds, *e.g.* the contribution of other tissues except the liver to the metabolism could be determined this way.

In the present study *in vitro* metabolic rates of TCBTs and PCBs were compared to determine differences in possible accumulation of both categories of compounds. Furthermore, CYPs involved in the metabolism of PCBs and TCBTs were identified by analysis of the correlations between specific CYP enzyme activities and the metabolic rates of individual PCB and TCBT congeners. When a specific CYP significantly correlates with the metabolic rate of a compound then it is likely that this enzyme is involved in its metabolism (Clarke, 1998; Parkinson, 1996). In order to increase the power of the correlation analysis, a broad range of CYP enzyme activities is needed. In the present study this condition was met by using rat hepatic microsomes from rats pretreated with agents that specifically induce CYPs. In addition, to confirm the involvement of certain CYPs, the effect of specific inhibitors on the metabolic rate was studied.

MATERIALS AND METHODS

Chemicals

TCBT isomers 3,3',4,4'-Cl_4-2-Me- (TCBT 87), 3,3'4,4'-Cl_4-5-Me- (TCBT 88), and 3,3',4',5-Cl_4-4-Me-tetrachlorobenzyltoluene (TCBT 94) were kindly provided by Prof. L. Brandsma from the department of Preparative Organic Synthesis of the Chemistry Faculty of Utrecht University. Their purity was >95 % as described by DeLang *et al.* (1998). The isomers are numbered according to Ehmann and Ballschmiter (1989). PCB 2,2',5,5'-TCB (PCB 52), 3,3',4,4'-TCB (PCB 77), 3,3',5,5'-TCB (PCB 80), 2,2',4,5,5'-PnCB (PCB 101), 2,3',4,4',5 PnCB (PCB 118), 3,3',4,4',5-PnCB (PCB 126), 2,2',3,3',6,6'-HxCB (PCB 136), 2,2',4,4',5,5'-HxCB (PCB 153), 2,2',4,4',6,6'-HxCB (PCB 155), 2,3,3',4,4',5-HxCB (PCB 157) and 2,2',3,4,4',5,5'-HpCB (PCB 180) congeners were obtained from Dr Ehrenstorfer (Augsburg, Germany). Their purity was >98 %. Analytical grade n-hexane, acetone, methanol, acetonitrile, and dichloromethane were obtained from J.T. Baker (Deventer, The Netherlands). Glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), and NADP were obtained from Boehringer (Mannheim, Germany). $MgCl_2$, K_2HPO_4, and NaH_2PO_4 were obtained from Merck (Darmstadt, Germany). β-naphthoflavone (BNF), isosafrole (ISO), clofibric acid (CLO), sodiumphenobarbital (PB), dexamethasone (DEX) , 8-methoxsalen (MOP), troleandomycin (TAO), α-naphthoflavone (ANF), ethoxyresorufin, methoxyresorufin, pentoxyresorufin, resorufin, and lauric acid, were obtained from Sigma (St. Louis, MO., USA). Orphenadrine (ORP) was purchased from Fluka AG (Basel, Switzerland).

Equipment

A HPLC system from Gynkotek with a P580HPG pump and a Basic Marathon autosampler from Separations (H.I. Ambacht, The Netherlands) was used, equipped with an UVD170S UV detector from Separations (H.I. Ambacht, The Netherlands), or a Kontron instruments SFM25 fluorescence detector from Beun de Ronde (Abcoude, The Netherlands). The HPLC was equipped with a Chromsep C18 column (200 × 3 mm i.d., 5 μm particles) preceded by a 10 mm guard column packed with the same material (Chrompack, Middelburg, The Netherlands). Spectrofluorimetry was performed using a Cytofluor 2300 (Millipore, USA) with excitation wavelength of 530 nm and an emission wavelength of 590 nm.

Animal treatment

Male and female CPB:uWU rats were obtained from the Central Animal Laboratory (Utrecht University). Rats were acclimatised for a week, prior to treatment. Male and female rats remained untreated or were dosed intraperitoneally for four consecutive days with the following CYP inducers, dissolved in corn oil or indicated otherwise: 40 (mg/kg/d) BNF (CYP1A1), 75 mg/kg/d PB in saline (CYP2B1/2), 230 mg/kg/d ISO (CYP1A2), 300 mg/kg/d CLO (CYP4A1), or 75 mg/kg/d DEX (CYP3A1/2). Livers of the individual rats were removed 24 hours after the last dose and perfused with ice cold saline until all blood was removed and stored at –80 °C till further use.

About 3 to 4 gram liver was used for the isolation of microsomes as described by Rutten *et al.* (1987). The protein content was determined in triplicate according to Bradford (1976) using a calibration curve with bovine serum albumin as a standard.

Microsomal CYP activities

The alkoxyresorufine O-dealkylase (=AROD) activity of the isolated microsomes were determined in triplicate, using ethoxyresorufin (CYP1A1), methoxyresorufin (CYP1A2) and pentoxyresorufin (CYP2B1/2) according to Burke *et al.* (1985). One mg microsomal protein was incubated with the alkoxyresorufin substrate in a 0.05 M Phosphate buffer (pH=8.0). The reaction started by adding 50 µl of a NADPH-regenerating system consisting of 1 µmol NADP, 5 µmol G6P, 0.95 Units G6PDH and 3 µmol $MgCl_2$.

Testosterone-hydroxylation assay and analysis of metabolites was performed as described by Wortelboer *et al.* (1990), using a NADPH regenerating system as described for AROD activities.

Lauric acid hydroxylation activity, which is indicative for CYP 4A1, was determined according to Jansen and DeFluiter (1992).

Characterisation of differentially induced microsomes

The inducing agents increased a broad range of enzyme activities as can be deferred from the relative induction of the indicative CYP activities (Table 1). These results are in coherence with earlier results (Wortelboer *et al.*, 1991).

Table 1. Enzyme activities of microsomes from untreated male (M) and female (F) rat and the relative induction of microsomes isolated after pretreatment with inducers.

Inducer	Enzyme activity	Specific CYP	Activity untreated rat (pmol.min^{-1}.mg protein^{-1})		Relative induction specific CYP activity	
			M	F	M	F
β-naphthoflavone	EROD	1A1	39	54	89	51
Isosafrole	MROD	1A2	50	56	82	18
Phenobarbital	PROD	2B1/2	7	5	14	7
Dexamethasone	Testosterone-15 β-hydroxylase	3A1/2	369	41	11	88
Clofibric acid	Lauric acid-12-hydroxylase	4A1	245	269	7	5

Characterisation inhibitors

Orphenadrine (ORP), 8-Methoxsalen (MOP), and Troleandomycin (TAO) were used to inhibit the activities of: CYP2B1/2 (ORP), CYP 2A (MOP) CYP3A1/2 (TAO), respectively (Koenigs *et al.*, 1997; Murray *et al.*, 1997; Reidy *et al.*, 1989; Watanabe *et al.*, 1999). Testosterone hydroxylation incubations were performed using microsomes from PB treated rats with different inhibitor concentrations (ORP 0-15-60-90-300 μM, MOP 0-5-15-30-60 μM and TAO 0-15-30-90-180 μM) to select the most specific inhibitor concentration. Incubations with 2 % (v/v) methanol were performed to eliminate possible solvent effects for ORP and TAO. ORP, MOP and TAO were pre-incubated for 15 minutes at 37 °C with microsomes from a male rat pretreated with PB in the presence of NADPH-regenerating system.

In vitro metabolism of TCBT and PCB

In vitro metabolic rate constants were determined according to (Boon *et al.*, 1998) and Kramer *et al.* (2000b) with minor adaptations. Testing the effect of protein concentration on the metabolic rate showed that metabolic rate was linear at concentrations over 0.5 mg/ml. To check the mass balance, PCB 153 (5 ng/μl) was added prior to the incubation. Incubations were performed in triplicate at 37 °C, using one mg protein in 0.95 ml 0.05 M phosphate buffer (pH=7.4) with a mixture of TCBT 87, 88, 94 or PCB 52, 77, 80, 101, 118, 126, 136, 155, and 180 at concentrations of 50 ng/ml. The metabolism started by adding 50 μl NADPH regenerating system (similar to testosterone assay). At 4 time points (t=0-150 minutes) 200 μl aliquots were pipetted in a 1 ml MeOH:buffer solution (85:15 v/v). TCBTs were extracted with 4 ml n-hexane (recovery >90 %) and the PCBs twice with 3 ml n-hexane (recovery >90 %). The organic phase was collected and 100 μl of

the volume standard (1.5 mg/l PCB 157) was applied. The samples were evaporated at room temperature to approximately 500 µl and were subsequently analysed.

Inhibition experiments were performed in triplicate at 37°C. 60 µM ORP or 30 µM TAO were preincubated in 1 ml 0.05 M phosphate buffer (pH=7.4), 1 mg microsomes from PB treated male rat and 50 µl NADPH regenerating system during 15 min. The metabolism of TCBT or PCB started by adding 5 µl of the TCBT or PCB solution (10 ng/µl acetone). 0.5 µM of ANF, a CYP1A2 inhibitor (Newton *et al.*, 1995) was co-incubated with TCBTs and PCBs without pre-incubation using microsomes from BNF pretreated male rat.

GC analysis

The samples were analysed on a Carlo Erba Mega 5360 HRGC-^{63}Ni ECD equipped with a 15 m DB5 column (ID 0.32 mm, film thickness 0.25 µm). TCBTs were analysed using split injection with a split ratio of 30 (Tinjector= 250 °C, Tcolumn= 250 °C). PCB samples were analysed using split-less injection (Tinjector= 250 °C) according to the following temperature program: 2 min at 70 °C, then to 255 °C at a rate of 20 °C/min, then 5 min at 255 °C, then to 280 °C at 30 °C/min, finally 4 min at 280 °C. Peak areas were determined.

Calculation of metabolic rate constant

Disappearance of parent compound relative to PCB 153 follows a first-order process. The ratio of the peak areas of the TCBTs or PCBs were logarithmically transformed and submitted to linear regression to time. The slope represented the metabolic rate constant (*k*; min^{-1}.mg $protein^{-1}$). Statistically significant ($p<0.05$) *k* values were used in the correlation analysis. Since the variation in the method is about 6 % (Kramer *et al.*, 2000b) an estimate of the lowest detectable *k* after 150 minutes was $4*10^{-4}$ min^{-1}. mg $protein^{-1}$. It has been observed that microsomes from PB pretreated rat can metabolise 8 µM PCB 153 at 1 pmol.min^{-1}.mg $protein^{-1}$ (Duignan *et al.*, 1987). At this concentration metabolism seems not to be saturated. Thus, at 250 nM the metabolic rate would be 0.03 pmol min^{-1}.mg $protein^{-1}$. This implies that after 150 min 5 pmol is metabolised. In this case the corresponding metabolic rate constant is smaller than the lowest detectable *k* of $4*10^{-4}$ min^{-1}.mg $protein^{-1}$. Thus, metabolism of PCB 153 is not detectable in our system. As a consequence, PCB 153 is considered useful to check the mass balance of the system.

The effect of an inhibitor on *k* was calculated according to equation 1

$$\% \ inhibition = \frac{k_{solvent} - k_{inhibitor}}{k_{solvent}} * 100\% \qquad \text{eq. 1}$$

Correlation analysis

CYP activities were correlated with *k* values for TCBTs and PCBs. Spearman's correlation coefficients were calculated using SPSS 7.5 for Windows (Chicago Il. USA) Correlations were considered to be statistically significant when $\alpha<0.05$.

RESULTS

Metabolic rate constants

In vitro metabolism of the TCBTs and PCBs could be described by first order kinetics as illustrated for TCBT 88; Microsomes from rats pretreated with phenobarbital increased the metabolic rate of TCBT 88 relative to microsomes of untreated rats (Fig. 1). Similar plots were obtained for the other congeners (plots not shown). Table 2 presents the first-order metabolic rate constants for the microsomal fractions of untreated and with inducer pretreated rats.

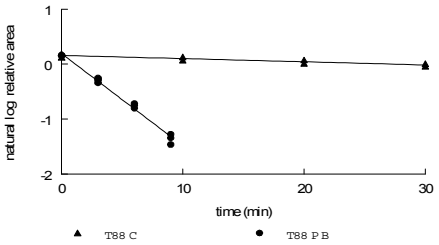

Figure 1. *In vitro* metabolism curves of TCBT 88 using rat hepatic microsomes of untreated (T88 C) and phenobarbital pretreated (T88 PB) female rats. The microsomal incubations were performed in triplicate.

Table 2. *In vitro* metabolic rate constants (*k*; min^{-1}.mg protein $^{-1}$) of three TCBTs, using microsomal fractions of untreated or treated rats of both sex. The metabolic rate constants of untreated rats are mean values and their standard deviations (Std) based on three rats. The other metabolic rates are mean values(N=3) and standard deviations (Std)

	TCBT 87		TCBT 88		TCBT 94	
			Male rats			
	k	Std	*k*	Std	*k*	Std
Untreated	0.015	0.001	0.018	0.005	0.044	0.001
BNF	0.029	0.001	0.029	0.022	0.074	0.002
CLO	0.015	0.010	0.013	0.009	0.051	0.012
DEX	0.120	0.005	0.107	0.007	0.351	0.001
ISO	0.049	0.003	0.035	0.004	0.322	0.012
PB	0.095	0.006	0.238	0.007	0.607	0.037
			Female rats			
	k	Std	*k*	Std	*k*	Std
Untreated	0.007	0.001	0.023	0.005	0.028	0.001
BNF	0.015	0.002	0.051	0.001	0.040	0.004
CLO	0.016	0.002	0.044	0.002	0.015	0.005
DEX	0.137	0.006	0.172	0.003	0.281	0.008
ISO	0.009	0.003	0.132	0.001	0.111	0.003
PB	0.081	0.001	0.292	0.004	0.809	0.076

A large variation in metabolic rate constants of the TCBTs was obtained by pretreating rats with the inducing agents (Table 2). The mean metabolic rate constants and standard deviation of untreated rats based on three rats were listed as well. TCBT 87 was metabolised at the lowest rate in microsomes of female rats.

Metabolic rate constants of the PCBs (Table 3) were about two to fifty times lower relative to the metabolic rates of the TCBTs, based on microsomal fractions of untreated rats. The pretreatment of rats with the different inducers resulted in a wide range of metabolic rate constants of PCB 52, 77, 101 and 136.

Table 3. *In vitro* metabolic rate constants for PCBs multiplied by 1000 (min^{-1}.mg $protein^{-1}$) using microsomal fractions of untreated or treated rats of both sex. The metabolic rate constants of untreated rats are mean values based on three rats. The other metabolic rate constants are mean values based on three determinations. Standard deviations are omitted for clarity reason.

Treatment	**Sex**	**di-*ortho* PCBs**					**Mono-*ortho* PCB**	**Non-*ortho* PCBs**		
		52	**101**	**136**	**155**	**180**	**118**	**77**	**80**	**126**
Untreated	F	3.2	1.1	2.7	0.9	-	-	-	1.3	-
	M	2.1	1.5	6.6	-	-	-	-	1.7	-
β-naphthoflavone	F	-	1.3	1.4	1.0	0.9	-	12.2	-	1.4
	M	-	1.3	4.9	1.5	1.5	-	8.5	1.6	-
Clofibric acid	F	4.3	-	1.3	-	-	-	-	-	-
	M	6.9	2.2	2.2	-	-	-	-	-	-
Dexamethasone	F	-	2.1	25.4	0.9	2.3	-	-	-	1.2
	M	7.1	2.1	31.4	0.9	-	-	-	-	-
Isosafrole	F	-	-	12.1	-	1.9	-	6.3	-	-
	M	-	24.3	74.9	1.4	1.3	-	17.9	3.0	1.1
Phenobarbital	F	10.2	3.9	43.5	1.0	2.3	-	-	-	-
	M	28.7	25.1	54.2	2.1	1.5	-	-	-	-

Correlation analysis

The results of the correlation analysis are presented in Table 4. Metabolism of the TCBTs was highly correlated with CYP 2B1 and 2B2 activity. Additionally, involvement of CYP2A1/2 or CYP3A1/2 was observed. The CYP2B1/2 correlated with the di-*ortho* PCBs 52 and 136. Additionally, CYP3A1 activity correlated with metabolism of PCB 136. Furthermore, PCB 155 correlated with CYP 2C11 activity. PCBs 77, 80, 126, and 180 did not correlate with any of CYP activities.

Table 4. Spearman's correlation coefficients ($p<0.05$) between *k* and CYP enzyme activities of TCBTs and PCBs. The combinations on which the correlations are based are tabulated (N). Statistically significant correlation coefficients are indicated in bold ($p<0.05$) or bold italic ($p<0.01$)

		CYP						
		2A1 2A2	3A1 3A2 (2A2)	2C11	2B1/2	2B1	1A1	1A2
	N	Testosterone metabolites				AR activities		
		7α	15β	2α	16β	Prod	Erod	Mrod
TCBT								
87	18	**0.46**	**0.52**	0.20	***0.76***	**0.50**	-0.06	-0.35
88	18	0.28	**0.72**	**0.57**	***0.69***	**0.54**	0.22	-0.20
94	18	***0.70***	0.39	-0.08	***0.75***	***0.70***	0.15	-0.10
PCB								
101	11	0.18	-0.47	-0.23	-0.28	-0.27	-0.19	0.39
126	3	0.50	0.50	0.50	-0.50	-0.50	0.50	0.50
180	7	-0.69	-0.25	-0.07	-0.40	0.40	0.49	0.29
80	4	-0.40	-0.20	-0.40	-0.40	-0.40	-0.27	-0.40
52	9	-0.37	-0.40	-0.33	***0.76***	**0.68**	-0.27	0.13
77	4	-0.40	-0.60	0.60	1.00	-0.40	-0.40	-0.40
136	18	-0.40	**0.51**	-0.25	**0.64**	**0.50**	-0.08	0.13
155	10	0.27	-0.15	**0.76**	0.28	-0.48	-0.76	-0.30

Characterisation inhibitors

Figure 2 depicts the most specific inhibitor concentrations as well as their inhibition percentage. TAO inhibited the formation of 15α-, 6β-, 15β- and 2β-OHT metabolites, reflecting CYP 3A1/2 and 2A1/2 activity. ORP reduced 16β-OHT formation for 80 % and to a lesser extent the formation of 2α-OHT and 16α-OHT, reflecting CYP2B1 and 2B2, as well as CYP2C11 activity. Some overlap between the inhibitory effect of ORP and TAO was observed by the inhibition of 15α-OHT formation. In addition, MOP was not selective since it inhibited the formation of nearly all OHT metabolites except for 15β and 2β OHT. Therefore MOP was not a useful inhibitor for the purpose of this study.

The inhibition of the metabolism of TCBTs and PCBs after co-induction with individual inhibitors is presented in Table 5. ORP, inhibits nearly 100 %, the metabolism of three TCBTs ($P<0.05$) and PCB 52, 101, 136 and 155, confirming the

involvement of CYP2B activity. TAO and ANF reduced the metabolic rates to a lesser degree.

Table 5. Inhibition percentages for TCBT and PCBs as determined by the *k* values for the TCBTs and PCBs with and without inhibitor but corrected for the solvent (demineralised water or MeOH). (* statistically sigificant $p<0.05$)

	TCBT 87	TCBT 88	TCBT9 4	PCB 52	PCB 77	PCB 101	PCB 136	PCB 155
ORP	95.4*	99.3*	100*	100*		100*	96*	100*
TAO	23*	1	26*	0		12.6	2.5	4.2
ANF					39.7*			

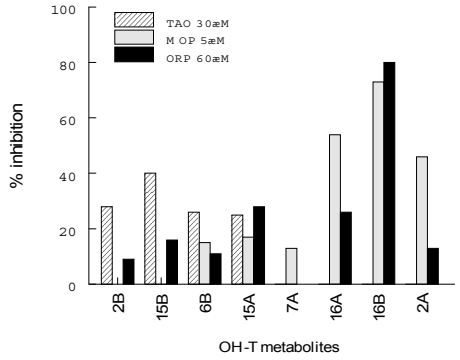

Figure 2. Effect of the inhibitors TAO, MOP and ORP at 30, 5 and 60 µM on the formation rate of OHT metabolites using microsomes from phenobarbital pretreated rats

DISCUSSION

Metabolic rate constants

In vitro metabolism of TCBT or PCB congeners using rat hepatic microsomes can be described by first order kinetics. This is in agreement with other *in vitro* studies, which reported first order kinetics for PCBs and TCBTs using rat hepatocytes or hepatic microsomes (Kramer *et al.*, 2000b; Mills *et al.*, 1985; Vickers *et al.*, 1986). In our study the disappearance of the parent compound was used as parameter to determine metabolic rate constants (k). To check the mass balance of the system the hardly metabolisable congener PCB 153 was added to the incubation (Muehlebach *et al.*, 1991; Vickers *et al.*, 1986). It has been demonstrated that microsomes from phenobarbital treated rats metabolised PCB 153 (Duignan *et al.*, 1987) at rates below

the level of quantification of the metabolic rate constants in our study, which was estimated at 0.0004 min^{-1}.mg $protein^{-1}$. Thus, during the time interval of the *in vitro* assay it is not likely that metabolism of PCB 153 influences the outcome.

The *in vitro* metabolic rate constants of the presently analysed PCBs were relatively low. This was expected because most of the congeners, such as PCB 52, 101, 118 and 180, are specifically used as indicators for exposure to PCBs (Prachar *et al.*, 1994) because of their known resistance toward metabolism. PCB 136, however, is known to be easily metabolised *in vivo* (Birnbaum, 1983) and *in vitro* (Schnellmann *et al.*, 1983). In the present study the k values for PCBs were 2 to 50 times smaller than for the studied TCBTs. The more rapid metabolism of these TCBTs would imply that the PCBs in our study could accumulate to larger extent, than TCBTs.

Correlation analysis

Using correlation analysis the simultaneous contribution of multiple enzymes to the metabolism can be identified at once, which is an important advantage of this method (Parkinson, 1996). In the present study it was observed that CYP2B1/2, CYP2A1/2, and CYP3A1/2 metabolised the TCBTs. Furthermore, these enzymes appeared to play a role in the metabolism of the di-*ortho* PCB 52 and 136 also. No correlations between k of PCB 77 and EROD or MROD were found, due to small number of correlations (table 4), however, BNF and ISO increased the metabolic rate constants. These chemicals specifically induce CYP1A1 and CYP1A2 (Parkinson, 1996). Inhibition by ANF showed that CYP1A2 is likely to play a role in the metabolism of this congener.

The identified enzymes CYP2B1 and 2B2 occur in the liver, lung and in the small intestines. The lungs contain 50% more and the small intestines 30% more than the liver, which contains 90.4 pmol.mg^{-1}protein (De Waziers *et al.*, 1990). These results suggest that the TCBTs and the di-*ortho* PCBs could be metabolised by these tissues as well. CYP1A1 (De Waziers *et al.*, 1990) and CYP3A1/2 (Debri *et al.*, 1995; Gushchin *et al.*, 1999) occur also in the small intestines suggesting that this tissue also contributes to the metabolism of the congeners. In general, exposure to PCBs occurs by oral ingestion. Thus, during the uptake from the GI tract CYP2B1/2, 1A1, 3A1/2 could already metabolise the TCBTs or the PCBs, suggesting lower bioavailabilities.

The involvement of CYP2B1/2 and CYP1A2 in the metabolism of the di-*ortho* substituted PCB 136 and the non-*ortho* substituted PCB 77, respectively, confirmed results obtained using purified CYP 2B1, CYP1A1, and CYP1A2 enzymes (Kaminsky

et al., 1981). CYP1A enzymes preferably metabolised non-*ortho* PCBs and CYP2B enzymes preferably metabolised di-*ortho* PCBs (Kaminsky *et al.*, 1981).

Since some CYPs show overlapping OHT formation, like CYP2A2 which forms 7αOHT and 15βOHT (Funae and Imaoka, 1987; Halvorson *et al.*, 1990), and mutual correlations between different OHT metabolites were observed, the interpretation of the results becomes complex. To confirm these correlations, CYP specific inhibitors are needed however, *e.g.* for CYP 2C11, 2A1 and 2A2 specific inhibitors were not available.

In conclusion, metabolism of the TCBTs in the present study is clearly faster than that of the PCBs. This suggests that the TCBTs might accumulate less in mammals than the PCBs. In addition, the TCBTs are more comparable with di-*ortho* substituted PCBs than non-*ortho* PCBs based on the CYP enzymes involved in their metabolism. CYP2B1 and 2B2 play a major role in metabolism of di-*ortho* PCBs and TCBTs, while non-*ortho* PCBs seem to be metabolised by CYP1A enzymes.

CHAPTER 4

METABOLIC RATE CONSTANTS OF UGILEC 141 ISOMERS AND POLYCHLORINATED BIPHENYL CONGENERS USING HUMAN HEPATIC MICROSOMES AND THE IDENTIFICATION OF INVOLVED CYTOCHROME P450 ENZYMES

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ABSTRACT

Ugilec 141®, a technical mixture consisting of tetrachlorobenzyltoluenes (TCBTs), was introduced as a replacement of polychlorinated biphenyls (PCBs) in the early 1980s. Accumulation of these TCBTs in the environment has been reported. In the present study metabolic rate constants of TCBT 87, 88 and 94 and PCB 52, 77, 80, 101, 118, 126, 136, 155 and 180 were determined by measuring the *in vitro* disappearance rates of parent compound using human liver microsomes (HLMs) from 17 donors. Additionally, HLMs were phenotyped for the major cytochrome P450 (CYP) activities: CYP1A2, CYP 3A4, CYP2C9, CYP2C19, CYP2B6, CYP2D6, CYP4A11. Correlation analysis of these data resulted in identification of the cytochrome P450 (CYP) enzymes involved in the metabolism of TCBT 87, 88 and 94 and PCB 77, 101, and 136. The identification of CYPs was further substantiated using specific CYP inhibitors. It was concluded that CYP2B6 plays a major role in the metabolism of TCBTs and the di-*ortho* substituted PCBs followed by CYP3A4. The non-*ortho* PCB 77 was mainly metabolised by CYP1A2. Furthermore, large interindividual variation in metabolic rate constants for each congener was observed, with variation coefficients of 20 to 137 %. Comparison of the metabolic rates of the TCBTs and the PCBs showed that TCBTs were metabolised 10 to 300 times faster than PCBs. Therefore TCBTs are expected to accumulate less in human tissue than PCBs.

INTRODUCTION

Ugilec 141®, was introduced on the European market in the early 1980s as an alternative for the use of polychlorinated biphenyls (PCBs). Ugilec 141 is a technical mixture of tetrachlorobenzyl toluenes (TCBTs) (Ehmann and Ballschmiter, 1989) and was used especially in hydraulic devices in mining industry. TCBTs are structurally and physico-chemically related to PCBs (VanHaelst, 1996). Shortly after their commercial introduction, TCBT levels up to 25 mg. kg^{-1} fish tissue were reported (Fuerst *et al.*, 1987b), indicating that TCBTs were able to accumulate in the food chain. For these reasons the European Union prohibited the application of Ugilec 141 in new machinery in 1994.

PCBs have been detected in many environmental samples including human tissue with high concentrations in adipose tissue. Elimination of PCBs from human tissue is slow as indicated by long half-lives, ranging from 124 to 338 days or from 2.6 to 4.8 years (Bruehler *et al.*, 1988; Phillips *et al.*, 1989). PCBs are predominantly eliminated from the organism by metabolism, which takes place primarily in the liver (Matthews and Dedrick, 1984). The rate-limiting step in the metabolism is a hydroxylation or oxidation reaction performed by cytochrome P450 enzymes (CYP) (Lutz *et al.*, 1977; Matthews and Dedrick, 1984)). CYPs are also involved in the metabolism of TCBTs as was shown *in vitro* using rat hepatic microsomes (Kramer *et al.*, 2000b).

Cytochrome P450 is an enzyme complex consisting of different enzymes (for review see Wrighton and Stevens (1992). The amount and the types of CYPs present in the liver depend on the genetic make up of the organism and external factors like diet or drug use (Wrighton and Stevens, 1992). The CYP composition of an individual liver determines finally the metabolic rates of xenobiotics. Therefore, it is important to investigate which specific enzymes are involved in the metabolism of xenobiotics.

The present study focussed on *in vitro* metabolic rate constants of TCBTs and PCBs by using human liver microsomes (HLMs). Three non-*ortho*, one mono-*ortho* and five di-*ortho* substituted PCBs were selected. Three TCBTs were chosen because theoretically these isomers could interact with the Ah-receptor (VanHaelst *et al.*, 1997) and as a consequence, could cause PCB and dioxin-like toxicity or biological effects (Safe, 1990; Safe, 1994). Furthermore, 17 different HLM samples were available to determine the interindividual variation of the metabolic rate constants for these

chlorinated substances. Moreover, the enzymes involved in the metabolism of TCBTs and PCBs were identified by correlation analysis (Clarke, 1998; Parkinson, 1996). To perform the correlation analysis, the individual HLMs were phenotyped by determining the catalytic activities of CYP1A2, CYP 3A4, CYP2C9, CYP2C19, CYP2B6, CYP2D6, CYP4A11, representing 60 to 80 % of the total cytochrome P450 enzymes in the human liver (Clarke, 1998; Gonzalez, 1992). In order to confirm the results obtained from the correlation analysis CYP activities were selectively inhibited.

MATERIALS AND METHODS

Chemicals

TCBT isomers 3,3',4,4'-Cl_4-2-Me- (TCBT 87), 3,3'4,4'-Cl_4-5-Me- (TCBT 88), and 3,3',4',5-Cl_4-4-Me-tetrachlorobenzyltoluene (TCBT 94) with a purity of >95 % (DeLang *et al.*, 1998) were kindly provided by Prof. L. Brandsma from the Department of Preparative Organic Synthesis of the Chemistry Faculty of Utrecht University. The isomers are numbered according to Ehmann and Ballschmiter (1989). PCB 2,2',5,5'-TCB (PCB 52), 3,3',4,4'-TCB (PCB 77), 3,3',5,5'-TCB (PCB 80), 2,2',4,5,5'-PnCB (PCB 101), 2,3',4,4',5-PnCB (PCB 118), 3,3',4,4',5-PnCB (PCB 126), 2,2',3,3',6,6'-HxCB (PCB 136), 2,2',4,4',5,5'-HxCB (PCB 153), 2,2',4,4',6,6'-HxCB (PCB 155), and 2,2',3,4,4',5,5'-HpCB (PCB 180) congeners were obtained from Dr Ehrenstorfer (Augsburg, Germany). Their purity was >98%. Analytical grade n-hexane, acetone, methanol (MeOH), acetonitrile were obtained from J.T. Baker (Deventer, The Netherlands). Glucose-6-Phosphate (G6P), and glucose-6-phosphate dehydrogenase (G6PDH) were obtained from Boehringer (Mannheim, Germany). $MgCl_2$, K_2HPO_4, and NaH_2PO_4 were obtained from Merck (Darmstadt, Germany). Lauric acid, bovine serum albumin, NADP, α-naphtoflavone (ANF), troleandomycin (TAO), caffeine, and testosterone, were obtained from Sigma (St. Louis, MO, USA). S-mephenytoine, nirvanol, and 4-OH-mephenytoine were purchased from Ultrafine chemicals (Manchester, UK). Orphenadrine (ORP) was obtained from Fluka AG (Buchs, Switserland). Tolbutamide was obtained from BUFA (Uitgeest, The Netherlands).

Human livers

Human liver tissue of 17 donors was obtained from redundant material after bi-partitioning of livers procured from multi-organ donors. The human livers were handled as described before (Olinga *et al.*, 1998). Consent from the legal authorities

and from the families concerned was solicited for the explantation of organs for transplantation purposes. The set of HLMs consisted of 9 livers from males with ages ranging from 2 to 51 with an average of 26.5. Eight female livers were obtained from donors with ages ranging between 6 and 53, with an average of 29.5. Human liver microsomes were isolated and stored until used according to Bouwman *et al.* (1992). The protein content was determined according to Bradford (1976) using a calibration curve with bovine serum albumin as a standard.

Phenotyping of HLMs

Specific CYP activities were determined in triplicate as specified in table 1 using HLMs.

Table 1. Overview of experimental conditions for the phenotyping assays

CYP	Substrate (Concentration μM)	Protein (mg/ml)	Incubation time (min)	Reference
1A2	Caffeine (500)	1.0	60	(Wolkers et al., 1996)
2C9	Tolbutamide (400)	1.0	60	(Zweers-Zeilmaker et al., 1996)
2C19,2B6	S-mephenytoine (800)	0.3	60	(Chauret et al., 1997; Lasker et al., 1998)
2D6	Dextromethorphan (500)	1.0	60	(Dayer et al., 1989)
4A11	Lauric acid (200)	0.5	10	(Jansen and DeFluiter, 1992)
3A4	Testosterone (300)	0.5	15	(Drenth et al., 2000; Purdon and Lehman-McKeeman, 1997)

HPLC Analysis

The analysis of the extracts were performed using a HPLC system from Gynkotek with a P580HPG pump, a UVD170S UV detector or a Kontron SFM 25 fluorescence detector, and Basic Marathon autosampler (Separations, H.I. Ambacht, The Netherlands). The samples of the testosterone, tolbutamide, and lauric acid incubation assays were analysed using a Chromsep C18 (200 * 3 mm ID, 5 μm particles) column preceded by a 10 mm guard column packed with the same material (Chrompack, Middelburg, The Netherlands). Dextromethorphan assay extracts were analysed using a Chrompack C18 Steel column (100 * 3 mm ID., 5 μm particles). S-mephenytoine assay samples were analysed using a Phenomenex C18 (150*4.60 mm) column preceded with a 30 mm guard column of the same material. The injection volume for each sample was 20 μl.

The HPLC methods are described in the references listed in table 1. Some minor modifications were applied as mentioned below. Tolbutamide assay extracts were analysed using a gradient mobile phase consisting of initially 85:15 10 mM sodium acetate (pH=4.3) and acetonitrile changing to 50:50 in 15 minutes at a flow rate of 2 ml/min. S-mephenytoine metabolites were analysed using a gradient mobile phase, consisting initially of 45% MeOH and 55% water and was brought in 20 min to 100% MeOH, at a flow rate of 1 ml per min.

In vitro metabolic rate assay

In vitro metabolic rate constants were determined according to Kramer *et al.* (2000b) and Boon *et al.* (1998). Incubations were performed in triplicate at 37 °C using 1 mg microsomal protein in 0.95 ml 0.05 M phosphate buffer (pH=7.4), with a mixture of TCBT 87, 88, and 94 or PCB 52, 77, 80, 101, 118, 126, 136, 155, and 180 at concentrations of 50 ng/ml. PCB 153 at a concentration of 50 ng/ml was added as internal standard. The reaction was started by adding 50 µl of a NADPH-regenerating system consisting of 1 µmol NADP, 5 µmol G6P, 0.95 Units G6PDH, 3 µmol $MgCl_2$. At each time point (t=0- 90 min.) 200 µl aliquots were pipetted in a 1 ml MeOH:buffer solution (85:15 v/v). The TCBTs were extracted with 4 ml n-hexane (recovery >90%). The PCBs were extracted twice with 3 ml hexane (recovery >90%). The organic phase was collected and 100 µl of a volume standard (1.5 mg/l PCB 157) was added. The n-hexane evaporated at room temperature to nearly dryness. Then the volume of each sample was adjusted to ca. 500 µl with hexane and subsequently analysed.

In vitro assay metabolic rate assay with inhibitors

Inhibition studies were performed in triplicate, using the inhibitors ORP, TAO and ANF (Guo *et al.*, 1997; Chang *et al.* 1994; Newton *et al.*, 1995). One mg HLM 11, because of its high metabolic activity toward the TCBT and PCB 136, was preincubated in 1 ml 0.05M phosphate buffer, with NADPH-regenerating system and 500 µM ORP or 20µM TAO for 15 minutes. Then 5 µl of the TCBTs or PCB 136 dissolved in acetone (10 ng/µl) was added to start the biotransformation reaction. PCB 77 was co-incubated with NADPH-regenerating system and 0.5 µM ANF using 1 mg HLM 8 because of its metabolic high activity toward PCB 77. The parent compounds were extracted as previously described and subsequently analysed.The percentage inhibition was calculated according to equation 1

$$\%\ inhibition = \frac{k_{solvent} - k_{inhibitor}}{k_{solvent}} * 100\% \qquad \text{eq. 1}$$

GC analysis

The samples were analysed on a Carlo Erba Mega 5360 HRGC-^{63}Ni-ECD equipped with a 30 m DB5 column (ID 0.32 mm, film thickness 0.25 μm). PCB and TCBT samples were analysed using splitless injection with the following temperature program: 2 min at 70 °C, 20 °C/min to 255 °C for 5 min, 30 °C/min to 280 for 4 minutes. The injector temperature was set at 250 °C. Splitless dosing times were 30 seconds preinjection and 99 seconds postinjection. The injection volume was 1 μl.

Calculation metabolic rate constant

Disappearance of parent compound relative to PCB 153 from the incubation mixture could be described by first order kinetics (eq. 2).

$$\frac{dA}{dt} = -k * A \qquad \text{eq. 2}$$

where A is the relative amount of TCBT or PCB to PCB 153, t is time (min) and *k* is the metabolic rate constant. The ratio of the peak areas of TCBT or PCB to PCB were logarithmically transformed and subsequently submitted to linear regression in time. The slope represented the metabolic rate constant. Only statistically significant ($p<0.05$) *k* values were used in the correlation analysis. An estimate of the detection limit of the *k* was obtained by calculating the minimal decrease of the relative amount of the parent compound based on two time the standard deviation of the t=0 samples after 90 minutes of incubation. This resulted in a lowest estimate of the metabolic rate constant $6*10^{-4}$ min^{-1}.mg protein^{-1}.

Correlation analysis

Spearman's correlation coefficients (S) between the CYP enzyme activities and *k* values for TCBTs and PCBs were calculated using SPSS for Windows (SPSS Inc. Chicago, USA). Correlations were considered statistically significant when $p<0.05$.

RESULTS

HLMs have been phenotyped for specific CYP activities. The results are listed in Table 2.

Table 2. Mean CYP activities (pmol/min/mg protein), standard deviation and the range of activities (min-max) of 17 HLM samples

Assay	CYP	Mean	Std	Min	Max
Tolbutamide-hydroxylase	CYP2C9	511	244	238	1154
Testosterone-6β-hydroxylase	CYP3A4	4650	3957	412	13052
Caffeine-N-demethylase	CYP1A2	41	26	6	86
Lauric acid-12-hydroxylase	CYP4A11	870	308	374	1452
Dextromethorphan-O-demethylase	CYP2D6	240	104	54	397
Nirvanol formation	CYP2B6	44	54	7	155
s-Mephenytoine-4-hydroxylase	CYP2C19	335	66	254	538

In vitro metabolism of the TCBTs by HLMs could be described by first order kinetics. The individual *k* values showed large interindividual variation, as indicated by the large variation coefficient (Table 3). The order of metabolic rate constants among the isomers is TCBT 94>TCBT 88>TCBT 87, which was statistically significant according to Wilcoxon sign test ($p<0.05$).

Compared to TCBTs metabolic rate constants of the PCBs were 10 to 50-fold lower (Table 3 and 4). Not all HLM fractions were able to metabolise PCBs to a measurable degree (estimated limit of quantification *k* <0.0006 min^{-1}).

Table 3. Mean metabolic rate constant (*k*) of the TCBTs based on different human liver microsomes with their corresponding variation coefficient (VC) and the minimum and maximum value of *k*. N is the number of HLMs.

TCBT	N	k (min^{-1}.mg $protein^{-1}$)	VC(%)	Min	Max
87	16	0.059	137	0.004	0.243
88	17	0.094	75	0.014	0.208
94	17	0.274	76	0.069	0.733

Strong correlations were found between CYP2B6 activity and the metabolic rates of the TCBTs (Table 5). To a lesser extent, *k* values of TCBT 88 and 94 correlated with CYP3A4 activity. The correlation between the *k* values of the individual isomers was high ($S>0.8$, $P<0.001$), indicating that the metabolism was catalysed by comparable CYPs. In addition, it appeared that enzyme activities mutually correlated; 6β-testosterone-hydroxylase correlated highly with nirvanol formation ($S=0.72$, $p=0.001$), with caffeine-N-demethylase ($S=0.67$, $p=0.003$), and with lauric acid-12-hydroxylase ($S=0.6$, $p=0.011$).

Table 4. Mean metabolic rate constant (*k*) of the PCBs based on different human liver microsomes with their corresponding variation coefficient (VC) and the minimum and maximum value of *k*. N is the number of HLMs

PCB	N	k (min^{-1}.mg $protein^{-1}$)	VC(%)	Min	Max
52	4	0.0068	79	0.0006	0.0133
77	17	0.0054	58	0.0009	0.0088
80	1	0.0011			
101	8	0.0010	40	0.0005	0.0012
118	3	0.0028	121	0.0008	0.0067
126	3	0.0014	20	0.0012	0.0017
136	10	0.0017	82	0.0006	0.0045
155	5	0.0035	68	0.0008	0.0040
180	5	0.0012	52	0.0009	0.0023

Table 5. Spearman's correlation coefficients (S) between metabolic rate constants of TCBTs and CYP activities based on human liver microsomes.

TCBT	CYP3A4	CYP1A2	CYP2D6	CYP4A11	CYP2B6	CYP2C19
87	0.45	0.23	0.30	0.28	0.87**	0.31
88	0.60*	0.46	0.24	0.38	0.93**	0.46
94	0.56*	0.45	0.37	0.35	0.85**	0.44

*p<0.05, **p<0.001

Metabolic rate constants of PCB 136 correlated statistically significantly with CYP2B6 and CYP3A4 activity (Table 6), while *k* values of PCB 77 correlated highly with CYP1A2 activity and to a lesser extent to CYP3A4 activity. For PCB 101 a correlation between *k* and CYP2C19 activity was observed. The statistically significant correlations of PCB 52, 118, 126, and 180 are based on a limited number of data and therefore considered of limited value.

Table 6. Spearman's correlation coefficients between *k* of PCBs and CYP activity

PCB	N	CYP3A4	CYP1A2	CYP2D6	CYP4A11	CYP2B6	CYP2C19
52	4	1.0*	0.00	0.20	-0.40	-0.60	1.0*
77	17	0.63*	0.92***	0.14	0.29	0.34	0.32
80	1	-	-	-	-	-	-
101	8	0.46	0.68	0.08	0.48	0.48	0.79*
118	3	0.50	-0.50	0.50	-0.50	-0.50	1.00*
126	3	-1.0	0.50	-1.0*	-0.50	-1.0*	-0.50
136	10	0.79**	0.33	0.006	0.006	0.94***	0.27
155	5	-0.56	-0.21	0.10	-0.21	0.67	0.21
180	5	-0.70	-0.60	-0.60	0.90*	-0.60	0.90*

*p<0.05, **p<0.01 ***p<0.001

Correlation analysis showed that CYP2B6, and CYP3A4 were the main enzymes involved in metabolism of the TCBTs and PCB 136, and CYP1A2 played a major

role in the metabolism of PCB 77. To confirm these observations, specific inhibitors were used to inhibit metabolism of the TCBTs or the PCBs. The results are presented in Table 7.

Table 7. Percentage inhibition of metabolic rate constants of the TCBTs and the PCBs after co-incubation with TAO, ORP or ANF (at 1 and 10 μM). The data represent the mean inhibition and the standard deviation based on three determinations.

Inhibitor	CYP	TCBT87	TCBT88	TCBT94	PCB77	PCB136
TAO	3A4	-18±9	-12±5	64±16*		96±23*
ORP	2B6	97±7.4*	80±21*	79±11*		100±11*
ANF (1 μM)	1A2				9±5	
ANF (10 μM)	1A2				85±9*	

*statistically significant ($p<0.05$)

ORP inhibited the metabolism of the TCBTs and PCB 136 almost completely (Table 7). These results confirmed that CYP2B6 indeed played an important role in the metabolism of these chemicals. TAO, which is a CYP3A4 inhibitor, markedly reduced the metabolic rate constant of PCB 136 and, to a lesser extent, TCBT 94. The inhibitory effect of ANF on the metabolic rate constant of PCB 77 became statistically significant at 10 μM, confirming the involvement of CYP1A2 in its metabolism (Table 6).

DISCUSSION

The TCBTs were much more rapidly metabolised than the PCBs. These results agree with earlier results based on rat hepatic microsomes using the same TCBTs and PCBs (Kramer *et al.*, 1999). Thus, humans also metabolise these TCBTs more easily than the PCBs. This suggests that TCBTs show a lower potential for accumulation in humans than PCBs.

The main conclusion from the correlation analysis was the involvement of CYP 2B6 in the metabolism of the three TCBTs and PCB 136. This was confirmed by the almost complete inhibition by ORP that inhibits CYP2B6 activity (Guo *et al.*, 1997). It has been observed that at the used ORP concentration approximately 20% of CYP3A4 activity was inhibited as well (Guo *et al.*, 1997). Additionally, involvement of CYP3A4 activity to the metabolism of PCB 136 and TCBT 94 was confirmed by the selective inhibitor TAO (Chang *et al.*, 1994; Newton *et al.*, 1995).

The contribution of CYP2B6 to the metabolism agrees with the results of a similar study using rat hepatic microsomes in which enzymes of the same family

(CYP2B1 and 2B2) were responsible for the metabolism (Kramer *et al.*, 1999). In our study metabolic rate constants of PCB 77 correlated highly with CYP1A2 activity. Inhibition by ANF, a selective inhibitor of CYP1A2 activity (Chang *et al.*, 1994; Newton *et al.*, 1995), confirmed this correlation. The presently obtained results agreed with studies on purified CYPs. CYP1A1 metabolised the planar non-*ortho* PCBs, like *e.g.* PCB 77 and CYP 2B1 metabolised *ortho*-substituted PCBs, like PCB 136 (Kaminsky *et al.*, 1981; Kennedy *et al.*, 1981; Matsusue *et al.*, 1996). Since metabolism of TCBTs correlates with CYP2B activity this suggests that these isomers resemble di-*ortho* PCBs, with respect to their structure. Consequently, this suggests that TCBTs have a bulky structure. This could imply that binding to the Ah-receptor, which requires planar compounds, is not very likely in contrast to what was suggested for these TCBTs (VanHaelst *et al.*, 1997).

The CYP activities of HLMs in our study, correspond with literature data (Chauret *et al.*, 1997; Dirven *et al.*, 1991; Eagling *et al.*, 1998; Gorski *et al.*, 1994). Therefore it can be anticipated that the HLMs in our study reflect hepatic enzyme activities of the general population. The mutual correlations suggest co-regulated which means that high enzymes activity of one CYP also implies high enzyme activity of the co-regulated one (Forrester *et al.*, 1992; Shimada *et al.*, 1994). In our study CYP3A4 consistently correlated with CYP2B6, which agrees with Shimada *et al.* (1994).

In conclusion, HLMs metabolised the TCBTs faster than the examined PCBs. These results suggest that accumulation of TCBTs in humans can be expected to be substantially lower than for PCBs. Furthermore, strong indications were obtained that CYP 1A2, 2B6 and 3A4 play a role in metabolism of TCBTs and PCBs. Within seventeen HLMs, large interindividual variation in metabolic rate constants was demonstrated. .

CHAPTER 5

PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL FOR TETRACHLOROBENZYLTOLUENES IN RAT: COMPARISON OF *IN VITRO* AND *IN VIVO* METABOLIC RATES

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ABSTRACT

Ugilec 141 is a technical mixture of tetrachlorobenzyltoluenes (TCBTs). It was introduced in the early 1980s as a replacement for polychlorinated biphenyls (PCBs). Based on physicochemical properties and accumulation in the environment, the use of this mixture was prohibited. To gain more insight in the toxicokinetics of these compounds in mammals, rats were exposed to a single intravenous bolus injection of a mixture of three TCBTs. At different time points after dosing the tissue and blood concentrations of the TCBTs were determined. The adipose tissue is the main storage compartment, followed by skin and muscle. The TCBTs were rapidly eliminated from the liver and the blood, with half lives ranging from 65 to 72 h. Additionally, the tissue concentration data for all three TCBTs were analysed using a Physiologically Based Pharmacokinetic (PB-PK) model. Sensitivity analysis illustrated that the elimination of the TCBTs was not influenced by metabolism only, but also by the blood flow through the liver. Furthermore, the metabolic rates derived from the model were compared to previously reported *in vitro* metabolic rate constants. The *in vitro* values for the TCBTs were only a factor 2 to 3 smaller than the *in vivo* metabolic rates, indicating the value of *in vitro* techniques for *a priori* parameterisation of PB-PK models.

INTRODUCTION

The technical mixture Ugilec 141 consists of tetrachlorobenzyltoluenes (TCBTs) isomers. It was developed in the early 1980s as a replacement for mixtures of polychlorobiphenyls (PCBs), especially in the mining industry. Similarities have been reported between TCBTs and PCBs with respect to physicochemical properties (VanHaelst, 1996), biochemical changes in mice (Murk *et al.*, 1991) and accumulation in fish (Fuerst *et al.*, 1987b; Wammes *et al.*, 1997). Due to the unwanted presence of Ugilec 141 in the environment and biota, the European Union prohibited the application of Ugilec 141 in new systems in 1994.

Presently, information of possible accumulation of TCBTs in mammals is limited. One study indicated that the mixture Ugilec 141 was eliminated faster from rat liver tissue than 2,2',4,5'5-PnCB (Bouraly, 1989).

Physiologically Based Pharmacokinetic (PB-PK) models have been used successfully to analyse the pharmacokinetic behaviour of lipophilic substances like PCBs and dioxins (Andersen *et al.*, 1993; Kedderis *et al.*, 1993; Lutz *et al.*, 1977; Lutz *et al.*, 1984). The parameters of such models may be obtained *a priori*, either from literature, *in vitro* studies, or by fitting the model to the experimental data or from *in vivo* experiments (Yang and Andersen, 1994). Extrapolation of *in vitro* metabolic rates resulted in good estimates for the *in vivo* metabolism of volatile and non-volatile organic chemicals (Carlile *et al.*, 1998; Houston, 1994b; Houston and Carlile, 1997; Krishnan and Andersen, 1994), but lipophilic substances, like TCBTs have not been studied in this respect.

The aim of the present study was to determine the kinetics of TCBTs and to determine the potential of these compounds to accumulate in mammals. Hence, rats were intravenously injected with a single bolus dose of a mixture of three TCBT isomers. The tissue concentrations were determined at various time points after dosing, and analysed using a PB-PK model. Furthermore, *in vitro* metabolic rate constants were compared to *in vivo* metabolic rate constants.

MATERIALS AND METHODS

TCBT isomers 3,3',4,4'-Cl_4-2-Me- (TCBT 87), 3,3'4,4'-Cl_4-5-Me- (TCBT 88), and 3,3',4',5-Cl_4-4-Me-Tetrachlorobenzyltoluene (TCBT 94) were kindly provided by Prof. L. Brandsma, from the department of Preparative Organic Synthesis of the

Chemistry Faculty of Utrecht University, The Netherlands. The isomers are numbered according to Ehmann and Ballschmiter (1989). The purity of the isomers is >95% (DeLang *et al.*, 1998). 2,2',4,4',5,5'-HxCB (PCB 153) was obtained from Dr Ehrenstorfer (Augsburg, Germany). The purity was >98%. Analytical grade n-hexane and acetone were obtained from J. Baker (Deventer, the Netherlands).

Animals

Adult male CPB:uWU rats (250-300 g) obtained from the Central Animal Laboratory Utrecht University were acclimatised for 5 days. Rats were housed individually in Macrolon III cages with filter top. Animals were maintained on a 12-hour light/dark cycle under conditions of constant temperature and humidity. The rats were provided with rodent chow and tap water *ad libitum*. After five days the rats were weighed and dosed intravenously with a mixture of: TCBT 87 (0.43 mg/kg), TCBT 88 (0.45 mg/kg), and TCBT 94 (0.48 mg/kg). The mixture was dissolved in a 20% tween/water (v/v) solution (polyethylenesorbitanmonooleate, Sigma, St. Louis, MO, USA) and administered via the tail vein at a constant volume of 1 ml/kg.

Animals were killed in groups of three over a period of 168 hours (t= 6, 10, 24, 48, 96 and 168 h) after sedation with CO_2 gas, which was followed by exsanguination by aorta punction. Blood was collected in glass tubes with EDTA as anticoagulant and stored at 4 °C until further use. About 1 to 4 g of liver, abdominal skin, abdominal adipose tissue, and muscle from the hind leg were collected. The tissues and organs were weighed and stored at -80°C until further use. Blood from the liver was removed by perfusing with saline.

Extraction of tissues

The frozen tissues and organs (except for blood samples) were freeze dried for 48 h. The dried samples were placed in a paper cartridge and spiked with 100 µl of PCB 153 (1.6 mg/l; internal standard) in n-hexane. Subsequently the samples were extracted with 80 ml n-hexane under reflux for 16 h (Bouraly, 1989). The recovery of the extraction was 90 ± 5.6 %. The extract was collected in pre-weighed flasks and the n-hexane was evaporated under nitrogen. The n-hexane extractable fat was then determined gravimetrically.

Clean-up

Glass columns (∅ 1 cm, 20 cm length) were filled with 4 grams Florisil (mesh 60-200 µm) deactivated with 5% water (w/w) to remove the n-hexane extractable fat

(Bouraly and Millischer, 1989). The column was pre-eluted with 5 ml n-hexane before adding 1 ml of the tissue sample containing maximally 100 mg fat extract. The samples were eluted with 30 ml n-hexane. The recovery of the clean-up over the column was 70 ±3.7 %. The eluates were concentrated prior to gas chromatographic analysis to a volume of ca. 500 µl.

Blood sample clean-up

Blood was extracted using 2 ml acetone and 2 ml n-hexane per gram blood (Boer and Wester, 1993). Prior to the extraction PCB 153 (1.6 mg/l) was added as an internal standard. The mixture was shaken for 30 seconds and centrifuged for 1 minute at 3000 rpm. The extraction was repeated three times. The organic phase was collected in pre-weighed vials and evaporated at room temperature under nitrogen. The n-hexane:acetone extractable fat content was determined gravimetrically. The recovery was 101 ±10 %.

GC-analysis

The extracts were analysed on a Carlo Erba Mega 5360 HR-GC-^{63}Ni-Electron Capture Detector equipped with a 15 m DB5 column (ID 0.32 mm, film thickness 0.25 µm). Samples were analysed using splitless injection according to the following temperature program. The analysis started at 70 °C, after 2 minutes temperature increased to 255 °C at 20°C/min temperature. After 5 min at 255 °C, temperature increased to 280 °C at a rate of 30 °C/min. After 4 minutes at 280 °C the analysis stopped. The injector temperature was set at 250 °C. Splitless closing times were 30 seconds preinjection and 99 seconds postinjection. The injection volume was 2 µl. The peak areas of the different compounds were determined relative to the peak area of PCB 153. Calibration curves for each compound using the internal standard PCB 153 (1.6 mg/l) were used to quantify the amount per sample. This amount was divided by the wet weight of the tissue or blood sample.

Model description

The general model structure (Fig. 1) was based on a model for PCBs (Lutz *et al.* 1977; Lutz *et al.* 1984).

The chemical uptake by the liver, and the remaining tissue, were modelled as a flow limited process. The tissue uptake by adipose tissue, muscle and skin was described by a diffusion limited process (Wang *et al.*, 1997). It was assumed that TCBTs are eliminated by first-order metabolism in the liver only.

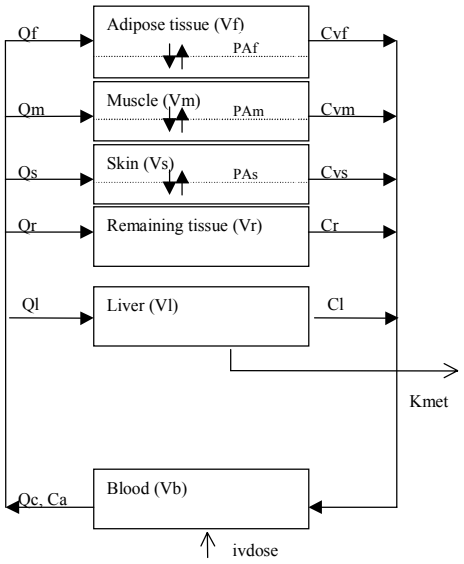

Figure 1. Physiologically based pharmacokinetic model for the kinetics of the TCBTs in rat. V, Q, C represent volume, blood flow and concentration of the different compartments, respectively. PAf, PAs, PAm represent the diffusional clearance.

Table 1. Physiological parameter values

Body weight (kg)	0.3	This study
Cardiac output (l/h)	6.08	Brown et al. (1994)
Fractional flow to tissues (% of cardiac output)		
Liver (Ql)	18.6	Brown et al. (1994)
Fat (Qf)	7.0	Brown et al. (1994)
Muscle (Qm)	27.8	Brown et al. (1994)
Skin (Qs)	5.8	Brown et al. (1994)
Remaining tissue (Qr)	40.8	Calculated
Tissue volumes (% of body weight) for rat		
Liver (Vl)	4.7	This study
Fat (Vf)	7.0	Brown et al. (1994)
Skin (Vs)	19.0	Brown et al. (1994)
Muscle (Vm)	40.4	Brown et al. (1994)
Blood (Vb)	7.4	Brown et al. (1994)
Remaining tissue (Vr)	13.5	This study
Tissue blood volumes (% of tissue volume)		
Fat (Vbf)	5	Kedderis et al. (1993)
Skin (Vbs)	2	Brown et al. (1994)
Muscle (Vbm)	4	Brown et al. (1994)

Model parametrisation

The physiological parameters are presented in Table 1. Tissue to blood partition coefficients (PC) were calculated according to Gallo *et al.* (1987). The PC for remaining tissue was set equal to the value for muscle.

The diffusional clearance constants for the adipose tissue (PAfC), muscle (PAmC), skin (PAsC), as well as the metabolic rate constant (K*met*) were optimised to the tissue and blood concentration data. A normalised sensitivity analysis was performed to determine sensitivity of the model forecast to the parameters (Evans *et al.*, 1994; Ploeger *et al.*, 2000).

Simulation software. The model equations (appendix A) were implemented in ACSL for Windows (version 11.4.1., MGA Software Inc. Concord. MA). Nelder-Mead (simplex) algorithm was used to optimise the model parameters in ACSL math (version 1.2, MGA Software Inc.).

RESULTS

The highest tissue concentrations were found in the adipose tissue followed by skin, muscle, liver, and blood for all three TCBTs (Fig. 2). For blood and liver, a rapid initial decline in tissue concentration was followed by a slower terminal elimination phase (Fig. 2), which could be described by a first-order process. The estimated terminal half-lives from the blood were 71, 62 and 65 h for TCBT 87, 88 and 94, respectively. All tissue concentration data were described adequately with the PB-PK model (Fig. 2 A, B, C) using the optimised parameter values (Table 2). Slight overestimation of the skin and muscle concentration of TCBT 87 and TCBT 88 between 6 and 48 hours after exposure was observed.

TCBT 87 deviated from the other two TCBTs with respect to tissue distribution as reflected in the PCs, metabolic rate and diffusional clearance. The partitioning to the liver (Pl) of TCBT 87 was approximately seven times higher than for TCBT 88 and 94. The relative distribution over the adipose tissue and the liver as indicated by the ratio Pf/Pl, appeared to depend on the isomer as well. The ratios were 12, 45 and 36 for TCBT 87, 88 and 94, respectively.

The metabolic rate constant of TCBT 87 was about 5 times lower than the metabolic rates of TCBT 88 and 94. The precision of the estimation of the metabolic rates, as indicated by the coefficient of variation (CV), ranged from 6-10 %, and was comparable among the isomers. The diffusional clearance constants for TCBT 87 in

each tissue tended to be higher than for the other two compounds. These estimates were less precise relative to the metabolic rate constants, for all three isomers, with CVs ranging from 0.5 to 35 %.

Table 2. Parameter values: calculated partition coefficients (Px), fitted diffusional clearance constants and fitted metabolic rate constants after *iv* injection.

	TCBT87	TCBT88	TCBT94
Partition coefficients *)			
Pl (Liver)	25	3.4	3.5
Pf (Fat)	300	152	125
Ps (Skin)	126	65	29
Pm (Muscle)	23	14	7
Pr (Remaining tissue)	23	14	7
Diffusional clearance constants (h^{-1})			
PAfC (Fat)	0.14 (0.36)$^{++}$	0.04 (7.50)	0.04 (0.50)
PamC (Muscle)	0.06 (18.33)	0.02 (35.00)	0.03 (4.66)
PAsC (Skin)	0.60 (16.66)	0.26 (0.77)	0.16 (18.75)
Metabolic rate constants (h^{-1})			
Kmet	2.1 (9.5)$^{++}$	10.3(5.8)	9.4 (8.5)

*) calculated as AUC_{tissue}/AUC_{blood}
$^{++}$ coefficient of variation (CV; %)

In vitro metabolic rate constants, as previously determined in hepatic microsomes from male rat (Kramer *et al.*, 2000b) were compared to *in vivo* metabolic rate constants (Table 3). The *in vitro* values were a factor 2 to 3 smaller than the metabolic rate constants, obtained after optimising the model to the *in vivo* data. Alternatively, optimising the diffusional clearance only, while fixing the metabolic rate to the *in vitro* values, overestimates systematically the tissue and blood concentrations (data not shown).

Table 3. Comparison of *in vitro* (K*in vitro*) and *in vivo* (K*met*) derived metabolic rate constants

TCBT	Kin vitro(h^{-1})*	Kmet (h^{-1})	Kmet/Kin vitro
87	0.96	2.1	2.2
88	3.78	10.3	3.3
94	4.14	9.4	2.2

from Kramer et al. (2000b)

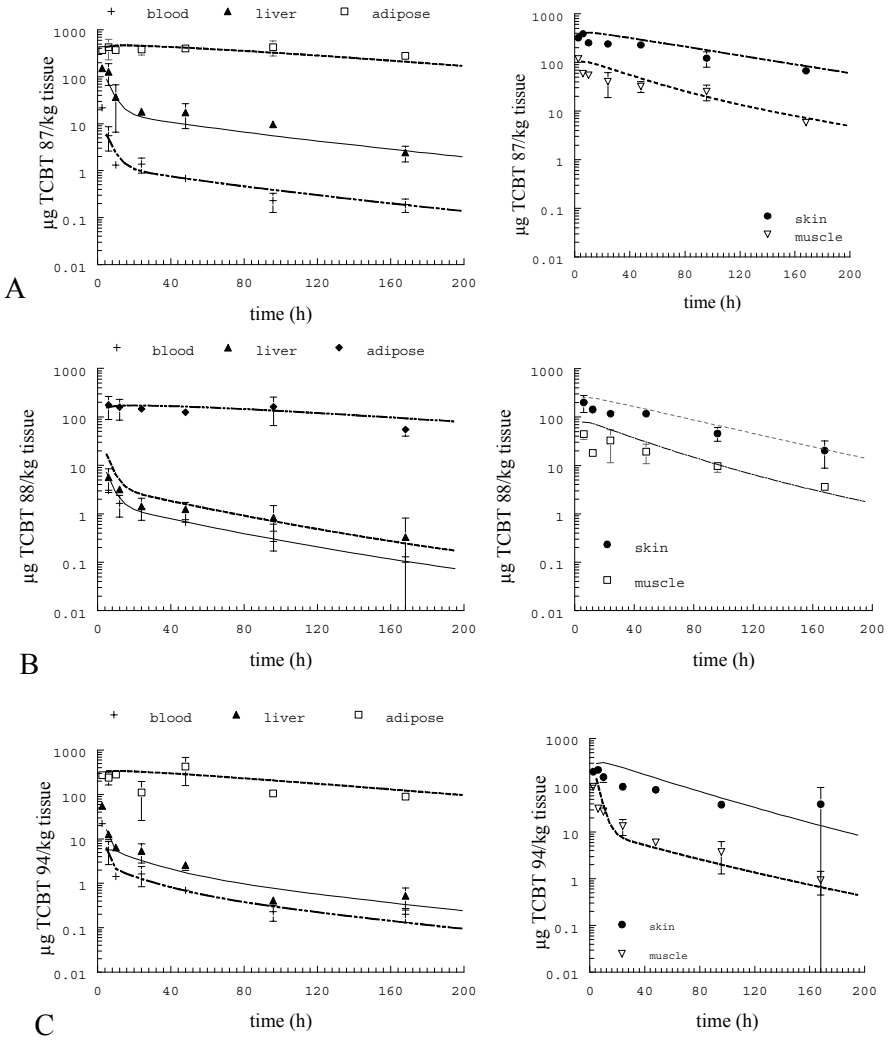

Figure 2. Concentration of TCBT 87 (A), TCBT 88(B) and TCBT 94 (C) in whole blood, liver, adipose tissue, skin and muscle of the rat. The lines represent the optimised model outcome. The scatter represent mean values and standard deviation (N=3).

The sensitivity of the PB-PK model forecast to the parameters is depicted in figure 3 and 4. It appeared that the blood concentrations were most sensitive to the metabolic rate (K*met*) during the distribution phase (Fig. 3). In addition, liver blood flow (Ql) was also a major determinant of blood concentration. An increase in the flow through the adipose tissue resulted in a slight increase in the blood concentrations (Fig. 3). Figure 4 shows that K*met* and the perfusion rate of the liver played a major role in the model predictions of the total amount metabolised. The sensitivity of the model to the diffusional clearance and the flow through the fat was only marginal as indicated by the small normalised sensitivity coefficients. Similar results from the sensitivity analysis were obtained for TCBT 88 and 94 (results not shown).

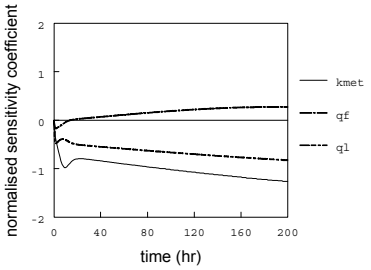

Figure 3. Sensitivity of model predictions to the metabolic rate (K*met*), and the perfusion rates of the adipose tissue (qf) and the liver (ql). The normalised predicted change in the concentration of TCBT 87 in blood concentration upon a 1 % change in the parameter values is plotted against time.

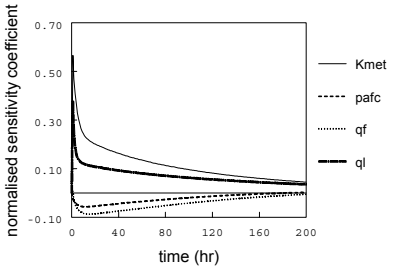

Figure 4. Sensitivity of K*met*, PAfC, Qf and Ql on the total amount metabolised using the PB-PK models for TCBT 87. The normalised predicted change in the total amount metabolised upon a 1 % change in the parameter values is plotted against time.

DISCUSSION

The present study showed that all three TCBTs reached the highest concentrations in the adipose tissue followed by skin, muscle, liver, and blood. This tissue distribution of TCBTs is comparable to that of PCBs and other lipophilic compounds in rat (Jordan and Feeley, 1999; Moir *et al.*, 1996; VandenBerg *et al.*, 1994). Furthermore, the first-order elimination of the TCBTs from the liver has also been observed for PCBs (Heinrich Hirsch *et al.*, 1997; Matthews and Dedrick, 1984; Ryan *et al.*, 1993). This confirms, in a qualitative way, our initial assumption about the comparability of the kinetics of PCBs and TCBTs. The uptake of TCBTs by the adipose tissue, muscle, and skin was presently modelled as a diffusion limited process, similar to previously reported models for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 2,3,7,8-tetrabromodibenzo-p-dioxin (Andersen *et al.*, 1997; Kedderis *et al.*, 1993; Wang *et al.*, 1997). Initially, Lutz and co-workers (1977) used relatively low blood flows for adipose tissue and muscle, *e.g.* 2.2 ml/min/100 g adipose tissue while Brown and co-workers (1994) reported a range of 18 to 48 ml/min/100g. A reduction of the blood flow resembled the effect of a diffusion limitation, but is not fully in agreement with the physiology. The diffusional clearance constants of closely related chemicals like for TCDD and tetrabromodibenzo-p-dioxin ranged between 0.08 and 0.1 for adipose tissue, for muscle between 0.03 and 0.05 and for skin between 0.015 and 0.09 (Andersen *et al.*, 1997; Kedderis *et al.*, 1993; Wang *et al.*,

1997). These parameter values agree with our results, suggesting a similar distribution process for TCBTs and dioxins.

Although the octanol to water partition coefficients of the TCBTs lie in the same range (Log K_{ow} 7.3-7.4), large differences in partition coefficients between the individual isomers were presently observed. TCBT 87 appeared to have the largest PCs for all tissues and the lowest fat to liver concentration ratio. The partitioning of TCBT 87 to the liver is relatively high (Pl=25), compared to Pls used in PB-PK models for related compounds. These Pls ranged from 3 to 17 (Kedderis *et al.*, 1993; Lutz *et al.*, 1977; Tuey and Matthews, 1980). TCDD and related compounds, like some PCBs, are selectively retained by CYP1A2 in the liver, causing alterations in the fat to liver concentration ratios, comparable to that observed for TCBT 87 (Andersen *et al.*, 1997; Andersen *et al.*, 1993; DeVito *et al.*, 1998; VandenBerg *et al.*, 1994). This suggests that the differences in tissue partitioning among the TCBTs, could be explained by selective retention of TCBT 87 by CYP1A2 in the liver.

TCBT 87 also differs from the other two isomers with respect to its metabolic rate. TCBT 87 was metabolised slower than TCBT 88 and TCBT 94. This agrees with the results of the *in vitro* study (Kramer *et al.*, 2000b). The present study showed that *in vitro* metabolic rate constants were 2 to 3-fold lower than the *in vivo* metabolic rates. This could be explained by the possible contribution of other tissues to the metabolism *in vivo*, since the *in vitro* data were based on metabolism in the liver only. However, differences in binding characteristics between *in vitro* and *in vivo* may be more likely, as was demonstrated for various drugs by Obach (1997). In this study, the extrapolation of *in vitro* data from experiments with microsomal fractions was improved when the free fractions of the substrate in the *in vitro* system and *in vivo* were taken into account. Thus, the factor of two to three between *in vitro* and *in vivo* might reflect the differences in the free fraction.

The direct insertion of these *in vitro* values in the PB-PK model resulted in an overestimation of the tissue concentrations.

Sensitivity of the metabolic rate constant on the model predictions of the amount metabolised showed that this parameter played an important role during the distribution phase of the TCBTs. This observation was substantiated by the almost similar terminal half-lives of the TCBTs, while the *in vivo* metabolic rate constants of the TCBTs differed maximally by a factor of five (Table 3). The blood flow through the liver also contributed substantially to the elimination as indicated by the

normalised sensitivity coefficient (Fig. 3). These findings suggest that other processes than the metabolism become rate limiting during the terminal elimination phase, like *e.g.* redistribution from the adipose tissue.

In summary, the *in vivo* kinetics of TCBTs were comparable to that of PCBs, with the highest tissue concentrations in the adipose tissue. Furthermore, it was demonstrated that the PB-PK model adequately described the tissue concentration data. This model showed that TCBT 87 was distinct from the other two TCBTs with respect to tissue partitioning and metabolic rates. Additionally, the model illustrated that during the distribution phase, metabolism played an important role in the elimination of the TCBTs, while during the terminal elimination phase possible other processes *e.g.* redistribution from the adipose tissue become rate limiting.

CHAPTER 6

PHARMACOKINETICS AND ORAL BIOAVAILABILITY OF TETRACHLOROBENZYLTOLUENES AND POLYCHLORINATED BIPHENYLS IN RATS

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ABSTRACT

Ugilec 141 is a technical mixture consisting of tetrachlorobenzyltoluenes (TCBTs). It was introduced in the 1980s as a replacement for polychlorinated biphenyls (PCBs). However, the TCBTs also accumulated in the environment. To determine the amount that enters the organism via ingestion of contaminated food, the oral bioavailability of TCBT 87, TCBT 88, TCBT 94 and PCB 77, PCB 101, and PCB 118 was measured in the present study. Rats were administered a mixture of these compounds by a single intravenous injection or a single oral dose. The calculated bioavailability for TCBT 88 and TCBT 94 was <20% and for TCBT 87 48%. The calculated bioavailability of the PCBs ranged from 60 to 100%. Two physiologically based pharmacokinetic (PB-PK) models, representing either uptake via the portal vein or via the lymphatic route were optimised to the oral tissue concentration data to estimate the fraction of the dose that was absorbed from the gastro-intestinal tract as well as the absorption rate constant. Both PB-PK models described the tissue concentration equally well. Modelling the uptake via the portal vein route resulted in slightly higher estimates of the fraction of the dose that was absorbed compared to that of the uptake via the lymphatic route. Additionally, an inverse correlation was observed between *in vitro* metabolic rates and calculated bioavailability. Thus, the low bioavailbilities of the TCBTs were suggested to be caused by first-pass elimination in the gastro-intestinal wall rather than by reduced absorption. In conclusion, the bioavailability of the TCBTs is lower than that of the PCBs.

INTRODUCTION

The technical mixture Ugilec 141 consists of tetrachlorobenzyltoluenes (TCBTs). It was developed in the early 1980s to replace mixtures of polychlorobiphenyls (PCBs). PCBs were widely spread in the environment, accumulated in biota and caused biological and toxic effects (Safe, 1993; Safe, 1994). Ugilec 141 is similar to mixtures of PCBs, with respect to their physicochemical properties (VanHaelst, 1996), accumulation in fish (Fuerst *et al.*, 1987b), and induction of enzyme activities in mice (Murk *et al.*, 1991). Due to these similarities with PCBs the use of TCBTs was prohibited in the European Union in 1994.

Exposure of mammals, including humans, to PCBs and TCBTs occurs in general via oral ingestion of food contaminated with these compounds. Absorption studies with PCBs have reported uptake efficiencies between 66 and 96 % in rat (Fries *et al.*, 1989; Tanabe *et al.*, 1981) and for humans between 27 and 88% (Schlummer *et al.*, 1998). These studies have also demonstrated that absorption of PCBs depends on molecular size, and number of chlorine atoms (Schlummer *et al.*, 1998; Tanabe *et al.*, 1981). Furthermore, it has been suggested that first-pass elimination could occur for rapidly metabolisable PCBs, reducing their bioavailability (Lutz *et al.*, 1977; Matthews and Anderson, 1975).

After absorption from the gastro-intestinal tract (G.I-tract) in dog and sheep, trichlorinated PCBs (Log K_{ow} 5-6) were taken up by the lymph (Busbee *et al.*, 1985; Ziprin *et al.*, 1980), whereas di-chlorinated PCBs (Log K_{ow} 5-5.4) were transported via the portal vein (Busbee and Ziprin, 1994). This difference was suggested to be due to the lower lipophilicity of the di-chlorinated biphenyls. The difference between both transport routes is that uptake via the lymph avoids first-pass elimination by the liver. This implies higher bioavailabilities (Charman *et al.*, 1997).

Absorption efficiencies or first-pass elimination of TCBTs have not been studied yet. It has been shown that some TCBTs are rapidly metabolised *in vitro* using hepatic microsomes from rat and human (Kramer *et al.*, 1999; Kramer *et al.*, 2000a). Compared to 3,3',4,4'-tetrachlorobiphenyl and 2,2',3,3',6,6'-hexachlorobiphenyl the *in vitro* metabolism of TCBTs was faster (Kramer *et al.*, 2000a). This rapid *in vitro* metabolism suggested the possible occurrence of first-pass elimination of TCBTs *in vivo* after oral exposure. First-pass elimination reduces oral bioavailability, resulting in lower tissue levels.

In this paper the oral bioavailability of three TCBTs and three PCBs were determined after exposing rats orally and intravenously to a mixture of these compounds. A physiologically-based pharmacokinetic (PB-PK) model was used to describe the bioavailability and the absorption rate of the compounds either following uptake via the portal vein route or via the lymphatic route.

MATERIALS AND METHODS

TCBT isomers 3,3',4,4'-Cl_4-2-Me- (TCBT 87), 3,3'4,4'-Cl_4-5-Me- (TCBT 88), and 3,3',4',5-Cl_4-4-Me-Tetrachlorobenzyltoluene (TCBT 94) were kindly provided by Prof. L. Brandsma, from the department of Preparative Organic Synthesis of the Chemistry faculty of Utrecht University. The isomers are numbered according to Ehmann and Ballschmiter (1989). The purity of the isomers is >95% (DeLang *et al.*, 1998). 3,3',4,4'-TCB (PCB 77), 2,2',4,5,5'-PnCB (PCB 101), 2,3',4,4',5-PnCB (PCB 118), and 2,2',4,4',5,5'-HxCB (PCB 153) were obtained from Dr Ehrenstorfer (Augsburg, Germany). Their purity was >98%. Analytical grade n-hexane and acetone were obtained from J. Baker (Deventer, the Netherlands).

Animals

Adult male CPB:uWU rats (250-300 g) obtained from the Central Animal Laboratory Utrecht University were acclimatised for 5 days. Rats were housed individually in Macrolon III cages with filter top. Animals were maintained on a 12-hr light/dark cycle under conditions of constant temperature and humidity. The rats were provided with rodent chow and tap water *ad libitum*.

After five days the rats were weighed and dosed orally or intravenously with a mixture of TCBTs and PCBs resulting in the following dose (mg/kg body weight): 0.43 mg TCBT 87, 0.45 mg TCBT 88, 0.48 mg TCBT 94, 0.19 mg PCB 77, 0.20 mg PCB 101 and 0.08 mg PCB 118. The mixtures for the oral study were dissolved in acetone. For each rat a single pellet was spiked with the dosing mixture 48 hours prior to the dosing. Rats were fasted for 24 hours before ingesting the spiked pellet. After the oral dosing food was provided *ad libitum*. The *iv* dose was dissolved in 20% tween/water (v/v) (polyethylenesorbitanmonooleate, Sigma, St. Louis, MO, USA) and injected in the tail vein at a constant volume of 1 ml/kg.

Animals from both the oral and *iv* study were killed in groups of three rats over a period of 168 hours (t= 6, 10, 24, 48, 96 and 168 h) after sedation with CO_2 gas, which was followed by aorta exsanguination. Kidney, skin, adipose tissue and blood

were collected from orally exposed rat. Adipose tissue, skin, muscle, kidney, liver and blood were collected from *iv* exposed rat. Tissue and blood extraction, clean-up and analysis were performed as previously described in Chapter 5. The samples were dissolved in n-hexane and analysed using HRGC- ^{63}Ni-ECD.

Model description

A PB-PK model, structurally similar to the model for TCBTs (Chapter 5), was applied to model the kinetics of the PCBs after intravenous dosing.

This PB-PK model of Chapter 5 was extended with oral exposure (Fig. 1). The absorption of TCBT or PCB was described as first-order disappearance of the dose from the GI-tract (*KA*) into the liver, representing uptake via the portal vein route (eq. 1) or into the venous blood compartment (eq. 2), representing the uptake via the lymphatic route.

$$\frac{dAl}{dt} = Ql(CA - \frac{Cl}{Pl}) - Kmet * Al + KAL * Dose * FL \qquad \text{eq.1}$$

Al denotes the amount in the liver, *t* is time, *Ql* is the blood flow through the liver, *CA* is the concentration of the compound in the arterial blood, C*l* is the concentration in the liver, *Pl* is the liver-blood partition coefficient, K*met* is the metabolic rate constant, KAL is the absorption rate constant from the GI tract via the portal vein, and FL is the fraction of the dose absorbed.

$$\frac{dAb}{dt} = Qv * Cv + KAS * FS * Dose - Qc * CA \qquad \text{eq.2}$$

Ab denotes the amount in the blood, *t* is time, *Qv* is the flow of the venous blood, *Cv* is the concentration in the venous blood, KAS is the absorption rate constant from the GI-tract to lymph, FS is the fraction of the dose that is absorbed, *Qc* is the cardiac output and *CA* is the concentration in the arterial blood.

Model parameterisation

Table 1 shows the physiological parameters. Tissue to blood partition coefficients (PCs) were calculated according to Gallo *et al.* (1987). Diffusional clearance constants (PAfC, PAmC, PAsC) and metabolic rate constants K*met* were optimised to the PCB tissue concentrations of the *iv* study.

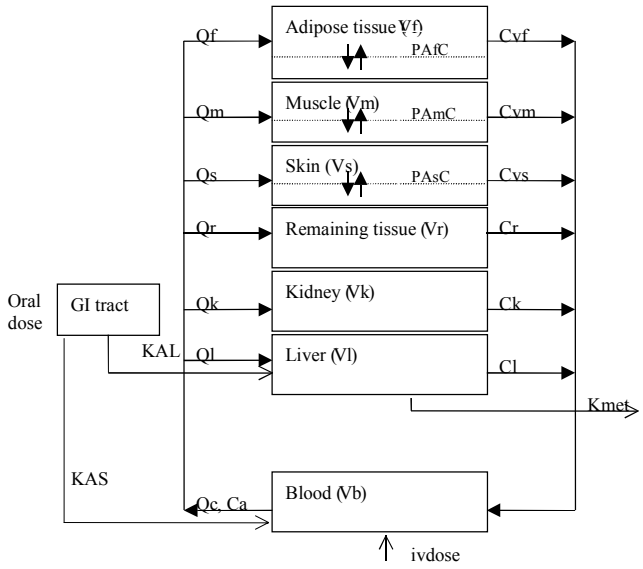


Figure 1. Physiologically based pharmacokinetic model for the TCBTs and PCBs in rat following uptake by the liver or by the lymph

Table 1 Model parameters

Body weight (kg)	0.3	This study
Cardiac output (l/h)	6.08	Brown et al. (1994)
Blood flow to tissues (% of cardiac output)		
Liver (Ql)	18.6	Brown et al. (1994)
Fat (Qf)	7.0	Brown et al. (1994)
Kidney (Qk)	14.0	Brown et al. (1994)
Muscle (Qm)	27.8	Brown et al. (1994)
Skin (Qs)	5.8	Brown et al. (1994)
Remaining tissue (Qr)	26.8	Calculated
Tissue volumes (% of body weight) for rat		
Liver (Vl)	4.7	this study
Fat (Vf)	7	Brown et al. (1994)
Kidney (Vk)	0.7	this study
Skin (Vs)	19	Brown et al. (1994)
Muscle (Vm)	40.4	Brown et al. (1994)
Blood (Vb)	7.4	Brown et al. (1994)
Remaining tissue (Vr)	13.5	This study
Tissue blood volumes (% of tissue volume)		
Fat (Vbf)	5	Kedderis et al. (1993)
Skin (Vbs)	2	Brown et al. (1994)
Muscle (Vbm)	4	Brown et al. (1994)

The bioavailability (FL or FS) and absorption rate constant (KAL or KAS) were obtained by fitting the PB-PK models for uptake via the portal vein route (eq. 1) or via the lymphatic route (eq. 2) to the tissue concentrations of the oral study.

The bioavailability (BA) was calculated according to eq. 3:.

$$BA = \frac{(AUC_{adipose} * Vf + AUC_{skin} * Vs + AUC_{blood} * Vb)_{oral}}{(AUC_{adipose} * Vf + AUC_{skin} * Vs + AUC_{blood} * Vb)_{iv}} \qquad \text{eq. 3}$$

where AUC is the area under the tissue concentration curve and V is the volume of the tissue (Table 1).

Simulation software. The model equations were written in ACSL for Windows (version 11.4.1., MGA Software Inc. Concord. MA) and are presented in the Appendix. For optimisation of the model parameters Nelder-Mead (simplex) algorithm was used of ACSL math (version 1.2, MGA Software Inc.).

RESULTS

Oral bioavailability of TCBTs

After oral ingestion of a food pellet contaminated with a mixture or the TCBTs (Fig.2), the highest concentrations were observed in the adipose tissue followed by skin, kidney and blood. Large differences in tissue concentration among the TCBTs were observed, although the rats were administered almost equal doses. The highest concentration in the adipose tissue was approximately 15 μg/kg tissue for TCBT 94, while for TCBT 87 the highest concentration in this tissue was approximately 300 μg/kg tissue (Fig. 2). Blood concentrations of TCBT 88 and 94 between 80 and 160 h after exposure, were close to the detection limit of 0.1 μg/kg.

The tissue concentrations of adipose tissue, skin and kidney were adequately described by the PB-PK model (Chapter 5) for uptake via the portal vein (Fig. 2, Table 3). The model underestimated the blood concentration and the kidney concentration of TCBT 94 (Fig. 2). The model for uptake via the lymph resulted in very similar fits (data not shown). This is illustrated by the almost equal weighed residual sum of squares from the model fits of both uptake models (Table 2).

Table 2. Overall Weighed Residual Sum of Squares of the PB-PK model for uptake via the liver and via the lymph of three TCBTs.

	TCBT87		TCBT88		TCBT94	
	Liver	Lymph	Liver	Lymph	Liver	Lymph
Overall	142	142	4.22	4.25	13.94	13.93
	PCB77		PCB101		PCB118	
	Liver	Lymph	Liver	Lymph	Liver	Lymph
Overall	15.02	15.04	3.63	3.68	9.62	9.04

The bioavailability (BA) of TCBT 87, 88 and 94 was 48, 20 and 6%, respectively (Table 3). The fraction of the dose that was absorbed as fitted by the lymphatic uptake model (FS) was comparable to the calculated BA. The fraction absorbed as fitted using the portal vein route was slightly higher compared to that absorbed via the lymphatic route. The absorption rate constants were independent of the uptake models.

Table 3. Fitted absorption rate constants (KAS or KAL) and bioavailability (FS or FL) of the TCBTs by uptake via either the portal vein route (L) or via the lymphatic route (S) and the calculated oral bioavailability (BA). The values between brackets represent the estimated coefficient of variation.

	TCBT 87	TCBT 88	TCBT 94
Calculated oral bioavailability			
BA*	0.48	0.20	0.06
Uptake via lymph			
KAS	1.45 (0.58)	0.55 (0.34)	0.57 (0.01)
FS	0.42 (0.10)	0.12 (0.10)	0.04 (0.01)
Uptake via portal vein			
KAL	1.84 (0.80)	0.55 (0.34)	0.58 (0.46)
FL	0.69 (0.10)	0.18 (0.10)	0.06 (0.14)

*BA was calculated according to eq. 3

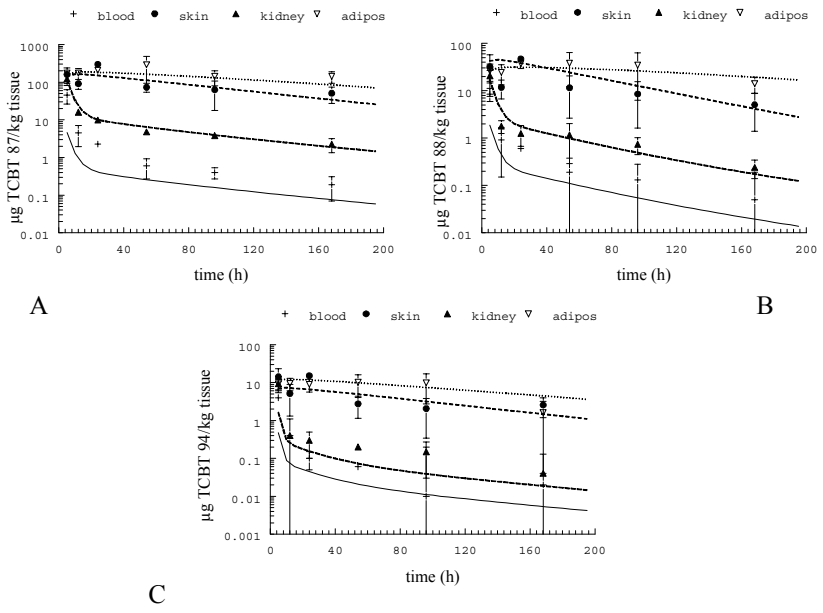


Figure 2. Rat tissue concentrations of TCBT 87(A), TCBT 88(B), and TCBT 94(C) after a single oral dose as described by the PB-PK model for uptake of TCBTs via the portal vein. The lines represent the optimised model simulation, the scatter represents the mean values of three determinations with standard deviation.

PB-PK model for the PCBs after intravenous dosing

The tissue disposition of the PCBs after the intravenous injection was comparable to that after oral exposure (Figs. 3 and 4). The highest concentrations were observed in the adipose tissue, followed by the skin, muscle, kidney, liver, and blood. The PB-PK model of the PCBs described the *iv* data adequately (Fig 3, Table 4).

Significant differences in metabolic rate constants (K*met*) among the PCBs were observed (Table 4). The lowest K*met* was found for PCB 118. The Pf of PCB 118 was also the lowest compared to that of the other two PCBs. The diffusional clearance constants of each tissue were comparable among the three PCBs. No differences in the coefficients of variation were observed among the optimised parameter values.

Table 4. Parameter values: calculated partition coefficients (Px), and fitted diffusional clearances (PAxC) and fitted metabolic rates (K*met*) after *iv* injection of rats with a mixture of PCBs using the PB-PK model

	PCB77	PCB101	PCB118
Partition coefficients *)			
Pl (Liver)	9	3	7
Pf (Fat)	112	75	35
Ps (Skin)	53	41	21
Pm (Muscle)	16	12	6
Pk (Kidney)	4	5	4
Pr (Remaining tissue)	16	12	6
Diffusional clearance constant (h^{-1})			
PafC (Fat)	0.05 (0.16)+	0.06 (0.13)	0.05 (0.20)
PamC (Muscle)	0.01 (0.20)	0.02 (0.20)	0.02 (0.25)
PasC (Skin)	1.0 (0.20)	0.20 (0.20)	0.85 (0.01)
Metabolic rate constant (h^{-1})			
Kmet	1.3 (0.03)+	2.5 (0.12)	0.2 (0.05)

*) calculated as AUCtissue/AUCblood
+) the coefficient of variation (%) is

Oral bioavailability of the PCBs

The optimised parameter values of the two oral PB-PK models of the PCBs representing portal vein and lymphatic uptake are presented in Table 5.

Table 5. Fitted absorption rate constants (KAS or KAL) and bioavailability (FS or FL) of the PCBs by uptake via either the portal vein route (L) or by the lymphatic route (S) and the calculated oral bioavailability (BA). The values between brackets represent the estimated coefficient of variation.

	PCB 77	PCB 101	PCB118
Calculated oral bioavailbility			
BA*	0.60	0.75	1.21
Uptake via the lymph			
KAS	0.41 (0.27)	0.27 (0.28)	0.25 (0.43)
FS	0.42 (0.12)	0.75 (0.09)	1.0 (0.33)
Uptake via the portal vein			
KAL	0.42 (0.28)	0.27 (0.29)	0.26 (0.44)
FL	0.47 (0.11)	0.82 (0.09)	1.0 (0.35)

*BA was calculated according to eq. 3

The fraction of the dose absorbed via the portal vein route (FL) was only slightly higher for PCB 77 and 101 compared to the uptake via the lymph. The calculated bioavailability (BA) of PCB 77 was 20 % higher than the fraction of the dose absorbed as estimated via the lymphatic route. No differences were observed between the absorption rates for both uptake models. Figure 5 shows that the PB-PK model for portal vein uptake, adequately described the tissue concentration data. Modelling the uptake via the lymphatic route resulted in similar model fits (data not shown).

This is illustrated by the almost equal weighed residual sum of squares of the model fits via both uptake routes (Table 2).

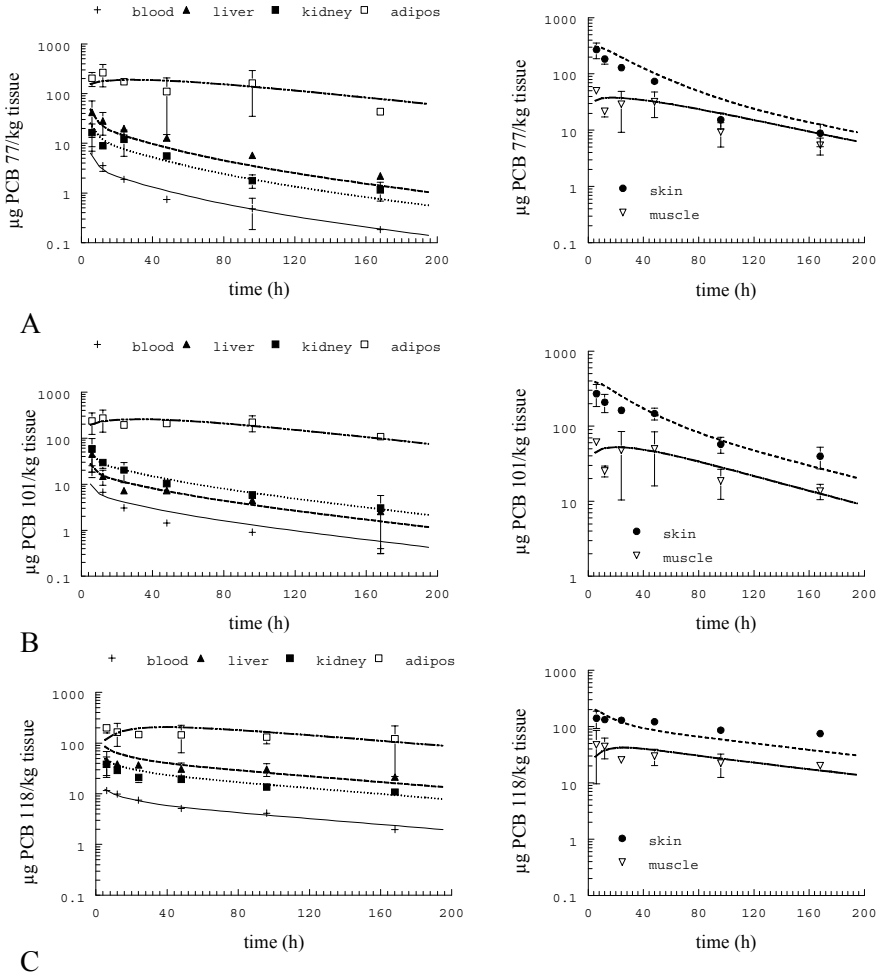


Figure 3. Tissue concentrations of PCB 77 (A), PCB 101 (B) and PCB 118 (C) after a single *iv* dose in rat as described by the PB-PK model. The lines represent the model simulation and the scatter represents the mean values of three determinations with standard deviation.

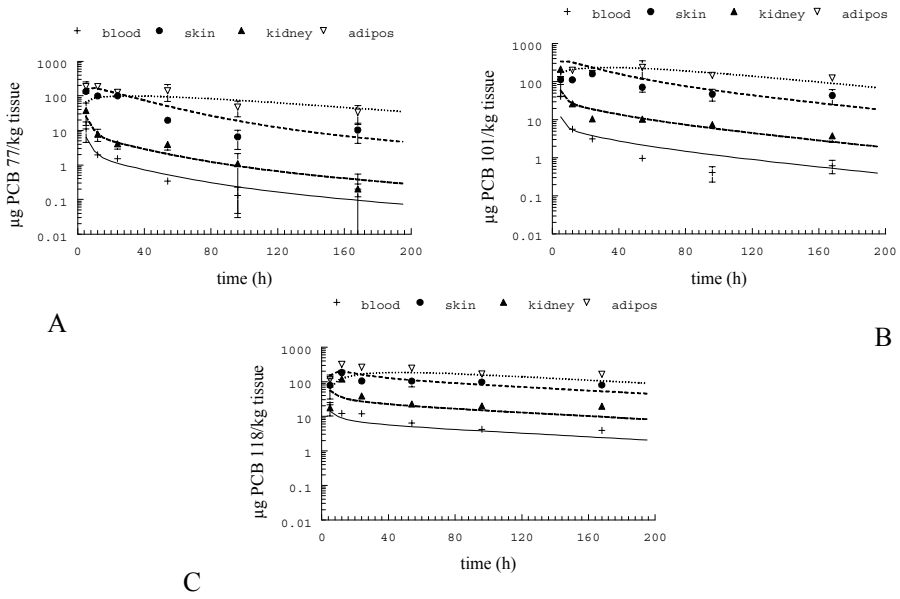


Figure 4. Tissue concentrations of PCB 77 (A), PCB 101(B), and PCB 118 (C) in rat, after a single oral dose as described by the PB-PK model for uptake of TCBTs via the portal vein. The lines represent the optimised model simulation, the scatter represents the mean values of three determinations with standard deviation.

DISCUSSION

The pharmacokinetics, including the bioavailability (BA) of three TCBTs and three PCBs, have been studied, by comparing tissue concentrations after an intravenous dose and an oral dose. The bioavailablilities of 6 and 20% for TCBT 94 and 88 respectively were low relative to TCBT 87 with a BA of 48%. The PCBs in our study showed a relatively high BA, ranging from 60 to 121%, which agreed with results for PCBs reported by Fries *et al.* (1989) and Tanabe *et al.* (1981). Thus, PCBs sharing similar lipophilicities, molecular size and almost the same degree of chlorination with TCBTs are efficiently absorbed over the GI-tract. Therefore it is anticipated that the low systemic oral biovailability of TCBTs is not likely to be caused by limited absorption. Thus, other factors must be identified to explain the presently observed differences in bioavailability between PCBs and TCBTs.

It appeared from the present study that modelling the uptake of the TCBTs and the PCBs via either the portal vein route or via the lymphatic route described the tissue concentration data equally well. Therefore, it was not possible to select the most appropriate model for the distribution of these chemicals after oral absorption. From a physiological point of view, the difference between FL and FS is the fraction of the dose that is metabolised during the first passage of the liver. Thus, in the case of high first-pass metabolism a larger fraction of the dose could be absorbed via the portal vein route (FL) relative to the lymphatic route (FS) to obtain similar blood and tissue concentrations. This would result in a large difference between FL and FS. In our study FL was only slightly larger than FS, indicating that first-pass elimination in the liver, is not very important. Hence, the most likely explanation would be metabolism by either the microflora in the G.I-tract, or by enzymes in the gut wall during the absorption phase.

When examining the bioavailability of the TCBTs and the PCBs as a function of their *in vitro* metabolic rate constants (Kramer *et al.*, 2000b), an inverse relation is observed *i.e.* the higher the *in vitro* metabolic rate constant, the lower the oral bioavailability *in vivo* (Fig. 5). These observations strongly suggest that these compounds could be degraded before reaching the systemic circulation.

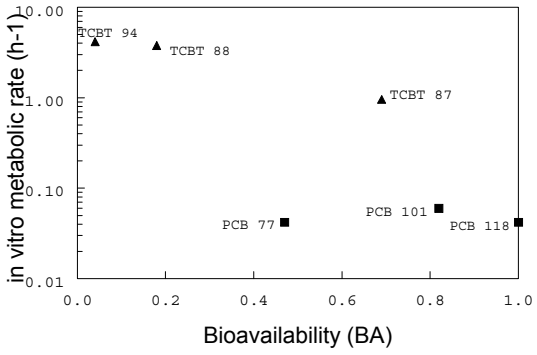


Figure 5. Relation between the calculated bioavailability and the in vitro metabolic rates from rat hepatic microsomes of the three TCBTs and the three PCBs

Degradation of TCBTs or PCBs by microflora has not been reported previously. Only for hydroxylated PCBs this type of degradation has been observed (Bergman *et*

al., 1982; Gustafsson *et al.*, 1981). Due to the limited data with respect to degradation of the PCBs and the TCBTs it was assumed that the contribution of possible degradation by the microflora was negligible. Thus, metabolism in the gut wall is the most probable explanation to explain the relatively low BAs for the three TCBTs.

Metabolism in the gut wall is likely to occur since the enterocytes contain cytochrome 2B1 and 2B2 that are involved in the metabolism of the TCBTs in rat (Chapter 3). These enzymes occur in the intestines at 1.3 times their amount in the liver. CYP1A1 and 1A2 are involved in metabolism of PCB 77, which also occur in the small intestine and the liver, respectively (De Waziers *et al.*, 1990). However, the contribution of other extrahepatic tissue, like lungs, can not be excluded.

In conclusion, it was observed that the three TCBTs and the three PCBs were rapidly absorbed from the GI-tract. Although the present results were not conclusive about the transport of the chemicals via either the portal vein or the lymphatic route, the low bioavailability of the TCBTs could be explained by first-pass elimination in the gut wall. The low bioavailability of the TCBTs implies that the accumulation after oral ingestion will be lower than for the structurally related PCBs.

CHAPTER 7

PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL OF TCBTs IN HUMANS, USING *IN VITRO* METABOLIC RATE CONSTANTS

H.J. Kramer, M.J. Zeilmaker, J.C.H. VanEijkeren, M. VandenBerg, and W. Seinen

ABSTRACT

Ugilec 141, a technical mixture consisting of tetrachlorobenzyltoluenes (TCBTs), was introduced on the European market in the early 1980s as a replacement for PCBs in hydraulic devices in the mining industry. From these systems TCBTs leaked into the environment and accumulated in fish. Since TCBTs are physicochemically related to the PCBs it was assumed that environmental problems would arise for TCBTs similar to that caused by PCBs, like food chain accumulation.

To estimate if humans could accumulate these TCBTs via their food like PCBs, a PB-PK model for TCBTs in rat was adjusted to the human situation. *In vitro* metabolic rate constants from human liver microsomes were extrapolated to *in vivo* values, taking into account the free fraction of the substrate and the specific intrinsic clearance. To compare the possible tissue levels of TCBTs with those of PCBs, single and multiple dose simulations were performed, assuming a daily dose of 89 ng/kg. This is an estimate of the average daily intake of total PCBs in the European Union. Simulated steady state levels of TCBTs were approximately 100 to 1000 times lower than PCB levels observed in human tissue. The estimated half-lives of the TCBTs ranged from 26 to 111 days. Based on the model estimations it was concluded that the TCBTs accumulate to much lesser extent in human tissue than PCBs.

INTRODUCTION

Ugilec 141 is a technical mixture consisting of tetrachlorobenzyltoluenes (TCBTs), which was used in hydraulic devices in mining industry because of its chemical stability. This highly lipophilic mixture was developed as a substitute for polychlorinated biphenyls (PCBs). PCBs are still ubiquitously present in the environment, and accumulate in the food chain, 30 years after the ban on their use. In top predators like *e.g.* humans high levels of PCBs have been measured, especially in the adipose tissue (Bachour *et al.*, 1998; Patterson *et al.*, 1994).

Physicochemically the TCBTs are related to the PCBs (VanHaelst, 1996), and also accumulate in the environment, including fish (Fuerst *et al.*, 1987b). This implies that humans could possibly accumulate these chemicals via consumption of fish. To elucidate whether this accumulation could occur, insight in the kinetics of these compounds is required.

Three isomers (Fig. 1) were supposedly ligands for the Ah-receptor based on geometric considerations (VanHaelst *et al.*, 1997). Interaction with this receptor is related to dioxin and PCB like toxicity. Thus, these three TCBTs could possibly cause dioxin-like toxicity.

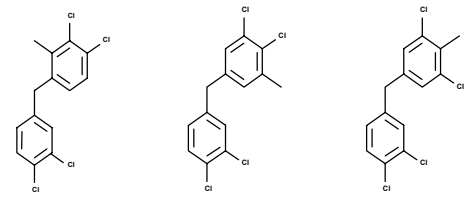


Figure 1. Chemical structure of three TCBTs. Numbering according to Ehmann and Ballschmiter (1989)

The pharmacokinetics of these three TCBTs in rats have been studied and described by a physiologically based pharmacokinetic (PB-PK) model (Chapter 5). The advantage of PB-PK models is that they can be scaled to *e.g.* other species like human by adjusting tissue volumes and blood flows (Krishnan and Andersen, 1994) and using either allometry or *in vitro* metabolic rate constants to scale metabolism to the relevant species. It appeared that *in vitro* metabolic rate constants in rat hepatic microsomes, lie in the same order of magnitude as the *in vivo* values, indicating the

value of *in vitro* methods to determine metabolism for PB-PK modelling (Chapter 5). Since *in vitro* metabolic rate constants in human liver were already determined (Chapter 4), these values were scaled to the *in vivo* situation, using the specific intrinsic clearance and the free fraction (Houston *et al.*, 1994b; Obach, 1997).

The aim of the present study was to elucidate whether TCBTs would accumulate to similar levels as PCBs at similar exposure levels. Hence, the available pharmacokinetic data on the TCBTs have been integrated in a PB-PK model for humans. With this model tissue concentrations have been estimated after a single dose and multiple doses using the total daily PCB exposure that has been estimated by Duarte Davidson and Jones (1994), because dietary levels of TCBTs have not been reported, except for fish. The simulated tissue levels of the three TCBTs were compared to PCB concentrations that have been analysed in human tissue (Juan *et al.*, 2000; Petreas *et al.*, 2000).

MATERIALS AND METHODS

The physiologically based pharmacokinetic model for TCBTs in the rat (Chapter 5) was scaled to estimate the pharmacokinetics in humans. Hence the tissue and organ volumes, blood flows and cardiac output were adjusted to a human adult male of 70 kg (Table 1). The model simulations were performed using ACSL for windows (version 11.4.1. MGA Software Inc. Concord. MA)

It has been demonstrated that tissue to blood partitioning in rat is lower than in humans for compounds with Log K_{ow} ranging from 1 to 4 (DeJongh, 1997). Compounds with a Log K_{ow} of 4 or more have almost equal partition coefficients (PCs) in both of these species. As TCBTs have a Log K_{ow} of about 7, it was assumed that the PCs for TCBTs between rats and humans are comparable (Table 2). Furthermore the diffusional clearance constants and the absorption rates from the PB-PK model of TCBTs in rats were applied directly (Table 2).

Table 1. Physiological parameters

Body weight (kg)	70	
Cardiac output (l/h)	342	(Brown et al., 1994)
Blood flow through tissue (% of cardiac output)		
Liver	22.7	(Brown et al., 1994)
Skin	5.8	(Brown et al., 1994)
Muscle	19.1	(Brown et al., 1994)
Adipose tissue	5.2	(Brown et al., 1994)
Remaining tissue	47.2	This study
Tissue volumes (% of body weight)		
Liver	2.6	(Brown et al., 1994)
Skin	3.7	(Brown et al., 1994)
Muscle	40.0	(Brown et al., 1994)
Adipose tissue	21.4	(Brown et al., 1994)
Blood	7.9	(Brown et al., 1994)
Remaining tissue	15.3	This study
Tissue blood volume (% of tissue)		
Adipose tissue	5	(Kedderis et al., 1993)
Skin	2	(Brown et al., 1994)
Muscle	4	(Brown et al., 1994)

Table 2 Partition coefficients of three TCBTs for different tissues and diffusional clearances used in the PB-PK model for TCBTs in humans, obtained from PB-PK model of TCBTs in rats.

	TCBT87	TCBT88	TCBT94
Partition coefficients			
Pl (Liver)	25	3.4	3.5
Pf (Fat)	300	152	125
Ps (Skin)	126	65	29
Pm (Muscle)	23	14	7
Pr (Remaining tissue)	23	14	7
Diffusional clearance constants (h^{-1})			
PAfC (Fat)	0.14	0.04	0.04
PamC (Muscle)	0.06	0.02	0.03
PasC (Skin)	0.60	0.26	0.16
Absorption rate constants (h^{-1})			
Ka	1.84	0.55	0.58
Bioavailability (%)			
BA	48	20	6

The *in vitro* metabolic rates using human liver microsomes were determined by measuring the disappearance of the parent compound (Kramer *et al.*, 2000b). This was modelled according to first order kinetics:

$$A(t) = A_0 \exp(-k_{vitro} t) \qquad \text{eq. 1}$$

where A represents the amount at time *t* and A_0 represents the amount substrate at t=0 and k_{vitro} represents the metabolic rate constant. In eq. 1 it is assumed that the time scale for diffusion is much smaller than the time scale for metabolism, which allows for a one-compartment modelling approach.

According to VanEijkeren (2000) k_{vitro} may be expressed as a function of a number of experimental parameters and some intrinsic hepatic properties:

$$k_{vitro} = \frac{f_m CL_m}{V_m + f_m / f_i \cdot V_i} = \frac{f_m CL_{m,s}}{1 + f_m / f_i \cdot V_i / V_m} \qquad \text{eq. 2}$$

where CL_m is the intrinsic clearance of the microsomes, and $CL_{m,s} = CL_m / V_m$ is the specific intrinsic clearance *i.e.* the actual metabolic rate by the enzyme, f_m is the compound's free fraction in the microsomes, f_i is the compounds free fraction in the incubation fluid, V_m is microsomal volume in the incubations tested and V_i is the volume of the incubation fluid (note that the sum of V_m and V_i equals the total volume of the *in vitro* system (V_{total}) in which metabolism is measured).

$CL_{m,s}$ and f_m are the parameters of interest. They were estimated by fitting eq. 2 to k_{vitro} data, which were obtained by varying the volume of the microsomal fraction V_m, while holding the total volume of the *in vitro* system constant (Fig. 2). Furthermore, eq. 2 was fitted to the k_{vitro} data assuming f_i to be 1 and V_i was substituted by $V_i = V_{total} - V_m$.

In the PB-PK model metabolism in the liver is modelled as:

$$\frac{dAl}{dt} = Ql(CA - \frac{Cl}{Pl}) - Kmet * Al \qquad \text{eq.3}$$

where *Al* is the amount in the liver, *Ql* is the blood flow, *CA* is the arterial blood concentration, *Cl* is the concentration in the liver, *Pl* is the liver to blood partition coefficient, and *Kmet* is the metabolic rate *in vivo*. For the *in vitro* to *in vivo* extrapolation, it was assumed that, in analogy to the *in vitro* system described above, *in*

vivo metabolism occurs by microsomes floating around in an "incubation" volume, *i.e.* the liver. This assumption allows a direct extrapolation of eq. 2 to the liver *in vivo*:

$$Kmet = \frac{f_m * CL_{m,s}}{1 + f_m / f_n * V_n / V_{m,l}} \qquad \text{eq.4}$$

where V_n is the volume of the non microsomal fraction and $V_{m,l}$ is the microsomal fraction of the complete liver, which is 45 mg per gram (Obach, 1997), f_n is the free fraction in the non microsomal fraction of the liver that is the only unknown parameter. Assuming that f_n is 1 for highly lipophilic substances like the TCBTs, then an upper limit of the *Kmet* could be estimated (see Table 3):

$$Kmet \leq \frac{f_m * CL_{m,s}}{1 + f_m * V_n / V_{m,l}} \qquad \text{eq.5}$$

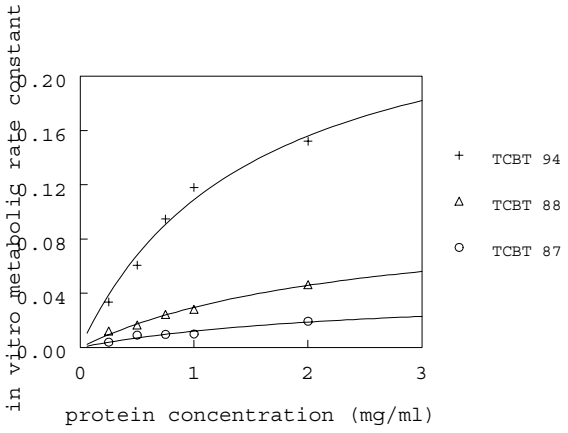


Figure 2. Effect of microsomal protein concentration on the metabolic rate constants (k_{vitro}) determined according to Kramer *et al.* (2000b) using human liver microsomes. The incubations were performed in 1 ml with a variable microsomal fraction. $CL_{m,s}$ and f_m were obtained by fitting eq. 2 to the *in vitro* metabolic rate constants. Each data point in the plot represents the mean value of three determinations.

Table 3 Estimated specific intrinsic clearance ($CL_{m,s}$), the free fraction in the human liver microsomes of TCBTs (f_m) and the estimate *in vivo* metabolic rate constant (*Kmet*), calculated according to eq. 5

TCBT	$CL_{m,s}$ (min^{-1})	f_m	Kmet (h^{-1})
87	16.8	0.0025	2.51
88	41.7	0.0024	6.05
94	181	0.0015	16.4

In chapter 6 it appeared that, on the basis of the available data, no distinction could be made between uptake via the liver or the lymphatic uptake route. Thus, in the present human model, the TCBTs entered the liver assuming transport via the portal vein.

The low bioavailability of the TCBTs in the rat has been suggested to be due to first-pass elimination in the gut wall (Chapter 6) by metabolism of TCBTs by CYP2B1, 2B2 and 3A1(Chapter 3). In human liver microsomes CYP2B6 and CYP3A4 are involved in metabolism of the TCBTs (Chapter 4), which are also expressed in human intestines (Gervot *et al.*, 1999; Zhang *et al.*, 1999). This suggests also first-pass elimination in humans, resulting in lower bioavailabilities. It was anticipated that the first pass elimination would compare to that in the rat (Table 2).

The model performed single dose and multiple dose simulations. During the exposure period it was assumed that the body composition did not change. Furthermore, the multiple dose model assumed daily dosing of 6.2 μg (= 89 ng/kg/d) based on a mean daily intake of total PCBs in Germany, Finland and the United Kingdom (Duarte Davidson and Jones, 1994) for a male adult of 70 kg.

RESULTS

Single dose

The tissue distribution and concentrations of all three TCBTs in humans after simulating a single oral dose were comparable (Fig. 3). An initial rapid decline in the TCBT concentration in the liver, blood and the remaining tissue was observed followed by a slower terminal elimination rate. The skin tissue reached the highest tissue concentration within a few hours followed by a rapid decrease and finally paralleled the terminal elimination rate of the blood and the liver (Fig. 3). The muscle

concentration decreased at a rather constant rate followed by the terminal elimination rate. TCBT 88 did not reach this rate after 2000 hours, while the other two did (Fig. 3). Furthermore very slow elimination of all three TCBTs from the adipose tissue was observed (Fig. 3).

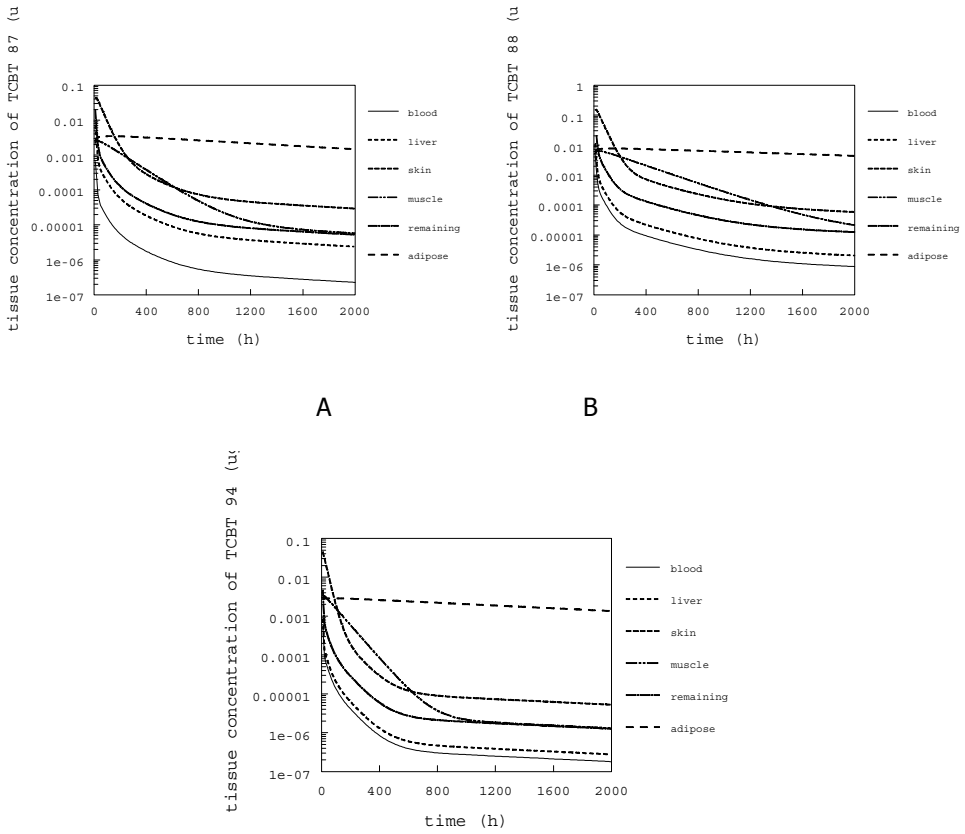
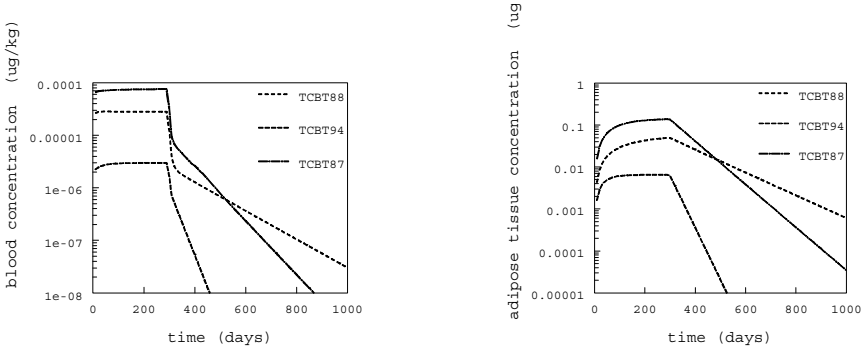


Figure 3 Tissue distribution in humans after a single oral dose of 89 ng/kg TCBT 87 (A), TCBT 88 (B) or TCBT 94 (C) as simulated using the PB-PK model for humans

Multiple doses

Steady state of the TCBTs in blood was reached within 10 days (Fig. 4A) similar to the skin, liver and muscle (data not shown), while steady state in the

adipose tissue was reached at least after 300 dosages (Fig.4B) for TCBT 94. TCBT 87 and 88 achieved steady state after approximately 500 and 800 days, respectively (data not shown). The concentrations in the adipose tissue under steady state conditions of TCBT 94, 88 and 87 were 0.003, 0.060 and 0.142 μg/kg adipose tissue, respectively. When dosing stopped after 300 days, the initial elimination rate from blood was high followed by a slower terminal elimination (Fig. 4A). Elimination from the adipose tissue paralleled the terminal elimination rate from the blood (Fig. 4B). The other tissues were comparable to the blood (results not shown). The terminal half-lives from the blood were 59, 111 and 26 days for TCBT 87, 88 and 94, respectively.



A B

Figure 4. Human blood (A) and adipose tissue (B) concentrations after 300 daily oral doses of 89 ng/kg of TCBT 87, TCBT 88 and TCBT 94 according to the PB-PK model for TCBTs in humans.

DISCUSSION

The pharmacokinetics of TCBTs in humans were estimated by adjusting the PB-PK model for TCBTs in the rat to the human physiology, with respect to blood flows tissue volumes and metabolism. The factor between the *in vitro* and *in vivo* metabolic rate constants in the rats was explained in terms of free fractions, specific intrinsic clearance and volumes as proposed by VanEijkeren (2000), Obach (1997) and Houston *et al.* (1994b). In the present study, these terms were quantified and used in the extrapolation to the *in vivo* situation, assuming a free fraction of 1 in the non-microsomal fraction (f_n) of the liver (eq.5). Table 4 shows, that under this assumption, the upper limit of the extrapolated *in vitro* metabolic rate constants from rat hepatic

microsomes were a factor 3 to 10 lower than *Kmodel*, representing the *in vivo* metabolic rate as obtained by fitting the PB-PK model to the tissue concentration data (Chapter 5). This factor agrees with Houston and Carlile (1997), who found an average difference between microsomal and *in vivo* clearance of a factor of approximately 5 for various drugs like diazepam, warfarin, and tolbutamide.

It appeared that, assuming f_n is 1, provides a reasonable upper estimate of the *in vivo* metabolism in rats (eq. 5) that is approximately one order of magnitude lower than the apparent *in vivo* metabolism (*Kmodel*; Table 4). Since no differences were seen between rat, human and other species with respect to free fractions in the microsomes (Obach, 1997), this assumption was also considered appropriate for the human hepatic microsomes. This would also provide an underestimate of the *in vivo* metabolism in humans. As a consequence, inserting this underestimate of the hepatic metabolic rate constant in the PB-PK model would overestimate tissue levels.

Table 4. Estimates of the free fraction of TCBT 87 and 94 in the microsomes (f_m) and the metabolic rates of the enzyme ($CL_{m,s}$) and the estimated *Kmet* in the rat using eq. 5. *Kmodel* represents the metabolic rate constant used in the PB-PK model for TCBTs in the rat

TCBT[#]	$CL_{m,s}$ (min^{-1})[+]	f_m[+]	*Kmet* (h^{-1})	*Kmodel* (h^{-1})*
87	1.20	0.0036	0.26	2.1
94	13.7	0.0039	3.19	9.4

* Chapter 5
[+] $CL_{m,s}$ and f_m in rat hepatic microsomes were obtained by fitting metabolic rate constants that were determined by increasing the microsomal fraction in the incubation (similar to Fig. 2)
[#] TCBT 88 no data available

One explanation for the difference between *in vitro* and *in vivo* values could be attributed partly to the contribution of extrahepatic clearance since the *in vitro* metabolic rates were based on the liver enzymes only. The enzymes CYP2B6 and CYP3A4 that metabolise the TCBTs (Chapter 4) are also detected in human lungs and intestines (De Waziers *et al.*, 1990). Thus it is likely that these tissues also contribute to the metabolism.

After approximately 300 to 800 daily dosages, the TCBTs reached steady state concentrations in the adipose tissue, which was the highest for TCBT 87 with 0.14 μg/kg. The accompanying steady state level in the blood was 0.07 ng /kg blood. In blood of five male adult volunteers from the United Kingdom assuming steady state, the levels of the very persistent PCBs 138, 153 and 180 ranged between 5 and 27 ng/g lipid, while levels of other PCBs were approximately 5 ng/g lipid (Juan *et al.*,

2000). Since the lipid fraction of human blood is approximately 0.5%, this implies levels of 25 to 135 ng/kg blood, which is approximately 300 to 2000 times higher than the model estimates for the TCBT blood concentration. Thus, the TCBTs have a two to three orders of magnitude lower potential to accumulate than the most persistent PCBs. This is due to the more rapid elimination of the TCBTs as indicated by the shorter terminal half-lives (26 to 111 days) compared to those of the PCBs which ranged between 2 to 6 years (Shirai and Kissel, 1996).

The bioavailability in humans was assumed to be analogous to that in the rat (Table 2) ranging from 6 to 48 %. Modelling the tissue concentrations, assuming 100 % bioavailability, increased the adipose tissue levels with a factor of approximately 2, 5, and 20, respectively, to 0.29 μg/kg for TCBT 87, and for TCBT 88 and TCBT 94 these levels would reach approximately 0.28 μg/kg and 0.05 μg/kg, respectively. Even these estimated levels are 30 to 2000 fold lower compared to PCB concentrations in adipose tissue of adult American women which ranged between 11 μg/kg and 110 μg/kg for individual congeners (Petreas *et al.*, 2000).

The steady state levels in the adipose tissue were higher than the dose (89 ng/kg/day). Expressing the tissue concentration at steady state relative to the dose provides a measure for the accumulation. Assuming 100 % bioavailability this would imply an accumulation factor of 3, for TCBT 87 and 88 and a factor of 0.5 for TCBT 94. Assuming bioavailability factors similar to rats the accumulation factor would become 1.5, 0.5 and 0.03 for TCBT 87, 88 and 94 respectively. Based on the adipose tissue levels of the PCBs (Petreas *et al.*, 2000) assuming that these levels were based on a daily intake of 89 ng/kg, the accumulation factor would range between 123 and 1235. Thus, TCBTs accumulate marginally compared to the PCBs.

The presented procedure to extrapolate *in vitro* metabolism to the *in vivo* situation for highly lipophilic substances like TCBTs provides reasonable estimates of the *in vivo* metabolism, under the assumption of rapid diffusional processes. Thus, this extrapolation procedure could be used to parameterise PB-PK models with respect to metabolism.

CHAPTER 8

GENERAL DISCUSSION

In the early 1970s, the use of polychlorinated biphenyls (PCBs) was prohibited because of their persistence in the environment and the accumulation in higher trophic levels of the food chain. Exposure to PCBs may elicited a variety of toxic and biological effects (Safe, 1994). Due to the regulations concerning the use of the PCBs, alternatives were developed. Ugilec 141 is one of the replacements of the PCBs. This technical mixture consists of tetrachlorobenzyltoluenes (TCBTs) and has similar physicochemical properties as the PCBs. A few years after the commercial introduction of the TCBTs in Europe, this PCB substitute was also found in tissues of aquatic species, like fish, at levels that compared to that of PCBs (Fuerst *et al.*, 1987b; Leonards and DeVoogt, 1989; Wammes *et al.*, 1997). Due to their physicochemical properties it was assumed that TCBTs could also accumulate in humans by consuming food contaminated with these substances and pose similar risks as PCBs. Hence, in 1994 similar regulations became effective in the European Union for TCBTs as for PCBs. These implied that in new machinery the use of Ugilec 141 was prohibited. However, from old devices the TCBTs could still leak into the environment and pose a possible health risk by accumulation in the food chain. The first objective of this thesis was to gain insight in the kinetics of TCBTs and

compare it with that of the PCBs, with special emphasis on possible food chain accumulation from fish to mammals, including humans. To achieve this, experiments were performed with three TCBTs and some PCBs that were tetra-, penta-, hexa- or hepta- chlorinated (PCB 52, 77, 80, 101, 118, 126, 136, 155, 180). Three TCBTs were selected out of the possible 96 isomers because of their supposed interaction with the Ah-receptor, which is a prerequisite to cause dioxin-like toxicity. The selected PCBs were closely related to the Ugilec 141 isomers with respect to the physicochemical properties, *e.g.* their lipid solubility as expressed by the octanol to water partition coefficient. Since metabolism was the rate-limiting step in the elimination of the PCBs, it was suggested that this would also be the case for the TCBTs. Thus, to determine possible accumulation of TCBTs and PCBs metabolic rate constants were determined *in vitro* using fish, rat, and human hepatic microsomes (Chapter 2, 3 and 4). Furthermore, *in vivo* studies have been performed to determine the tissue distribution of the selected TCBTs and PCB 77, 101 and 118 (Chapter 5 and 6) after intravenous or oral exposure. With physiologically based pharmacokinetic (PB-PK) modelling the kinetics of these TCBTs and PCBs were quantitatively described. The information from the chapters 2, 3, 4, 5 and 6 was integrated in a PB-PK model to estimate the tissue disposition in humans (Chapter 7).

The second goal was to identify the cytochrome P450 (CYP) enzymes involved in the metabolism of the selected TCBTs and PCBs (Chapter 3 and 4), to eventually indicate the possible contribution of extrahepatic tissue to the metabolism.

One of the central themes in human toxicology is the validation of methods that are used to extrapolate experimental data obtained from experimental animals, or from *in vitro* studies to the human situation. In our study *in vitro* metabolic rate constants obtained from rat and human liver microsomes (Chapter 2, 3, and 4) were extrapolated to *in vivo* values for the use in a rat and human PB-PK model for TCBTs (Chapter 7). Thus, the third goal of this study was to evaluate the relationship between *in vitro* and *in vivo* values and the merit of these *in vitro* parameters in PB-PK modelling (Chapter 5).

KINETICS OF TCBTs

Absorption

The presently observed high bioavailabilities of PCB 77, 101 and 118 in our study, ranging from 60 to 100% (Chapter 6), indicate their efficient absorption from

the GI-tract, which agrees with absorption efficiencies reported earlier (Fries *et al.*, 1989; Tanabe *et al.*, 1981). In general, the absorption efficiency of PCBs depends on lipid solubility and the molecular size (Tanabe *et al.*, 1981). Although the bioavailability of the individual TCBTs was relatively low (between 6 and 48%), it was expected that the TCBTs, with comparable lipid solubility and molecular size, would be absorbed from the gut with comparable efficiencies as PCBs (Chapter 6). After the absorption, PCBs and other highly lipophilic substances *e.g.* DDT and dioxins sequester in chylomicrons in the lymph and are transported to the thoracic duct where lymph mixes with the venous blood (Busbee *et al.*, 1985; Charman *et al.*, 1997; VandenBerg *et al.*, 1994). The PB-PK model for oral uptake including either transport via the lymphatic route, or via the portal vein route, did not provide statistical evidence to express preference for one of both models (Chapter 6). Possible transport via the lymphatic route has some kinetic implications that will be discussed in the metabolism section.

Tissue distribution and disposition

The tissue distribution of TCBTs and PCBs after a single bolus injection of a mixture of TCBTs and PCBs (Chapter 5 and 6) in rat showed comparable patterns and agree with literature data for PCBs, dioxins and other polyhalogenated hydrocarbons (Kedderis *et al.*, 1993; Matthews and Dedrick, 1984; VandenBerg *et al.*, 1994). In the rat the adipose tissue is the main storage site followed by skin and muscle. After an initial distribution to the well-perfused liver the TCBTs redistribute to the skin, muscle, and adipose tissue (Chapter 5 and 6). In humans however, the PB-PK model simulations of the tissue concentrations after a single dose (Chapter 7) showed that initially the skin is the main storage compartment followed by the adipose tissue. This disparity, with respect to the tissue distribution in rats, is likely due to the larger adipose tissue depots (21 %) in humans compared to the rat (7 %). Since the fat volume is larger in humans the concentrations, after equal dosing, will be lower compared to the fat tissue of the rat.

The tissue distribution of the TCBTs in rats illustrates that the lipophilicity of these chemicals is the major determinant for the tissue disposition. However, the isomer specific tissue distribution of the TCBTs indicates that other factors beside lipophilicity influence this process (Chapter 5). Most pronounced was the 7 fold higher liver to blood partition coefficient of TCBT 87 relative to that of the other two TCBTs. Such selective liver retention has been observed previously for polyhalogenated aromatic hydrocarbons like 2,3,7,8-TCDD or PCB 126. This

retention was due to binding to CYP1A2 in the liver, resulting in relatively high liver to blood partition coefficients (DeVito *et al.*, 1998; VanBirgelen *et al.*, 1994a). A planar structure appeared to be a prerequisite for this binding to CYP1A2 (DeVito *et al.*, 1998; VanBirgelen *et al.*, 1994b). Theoretically, TCBT 87 and 88 were the most likely structures to adopt a planar conformation (VanHaelst, 1996), however, based on the enzymes that metabolise the TCBTs in rats and humans, it was suggested that these isomers most probably have a non-planar conformation (Chapter 3 and 4). Since neither TCBT 88 nor TCBT 94 elicited a preference for the liver, the conformation of TCBT 87 might thus explain the disparity in the liver to blood partitioning. Thus possibly the methyl-group in the *ortho* position might determine the selective retention by the liver.

Metabolism

Different species

Metabolism of the TCBTs, similar to the PCBs, is considered to be the rate-limiting step in the elimination of these compounds. Rat hepatic microsomes metabolised the TCBTs 2 to 50 times faster than the selected PCBs (Chapter 3), while human hepatic microsomes metabolised the TCBTs even 10 to 300 times faster than the selected PCBs (Chapter 4). Trout hepatic microsomes with their relatively low concentrations of biotransformation enzymes and the relative low metabolic activity compared to mammals (Sijm and Opperhuizen, 1989), metabolised neither the TCBTs (Chapter 2) nor the PCBs to a detectable rate within in the observed time-span (Murk *et al.*, 1994; White *et al.*, 1997). Thus, these results imply that mammals eliminate TCBTs faster than PCBs, while in fish elimination was expected to be equally slow for both categories of compounds. Based on these results, we suggested that the body burden of TCBTs in fish would be comparable to the body burden of PCBs. This is substantiated by the finding of comparable tissue levels in the eel (Wammes *et al.*, 1997). In mammals, however, due to the fast metabolism of the three TCBTs, the body burdens of these substances are suggested to be lower than for the PCBs.

Role of Cytochrome P450 enzymes

Various CYP enzymes appeared to metabolise the three TCBTs and some PCBs in both rat and human liver (Chapter 3 and 4). Table 1 summarises the main results.

Table 1. Overview of the major enzymes involved in the metabolism of the TCBTs and the PCBs using hepatic microsomes

	Rat	Human
TCBT 87,88, 94	CYP2B1, 2B2, 3A1, 3A2	CYP2B6, 3A4
PCB 52, 101, 136	CYP2B1, 2B2, 3A1, 3A2	CYP2B6, 3A4
PCB 77	CYP1A1, 1A2	CYP1A2

Isomer dependent metabolism was observed for the three TCBTs in Chapter 2, 3, and 4. TCBT 94 was metabolised faster than TCBT 88 and TCBT 87 in both rats and humans. Since the same CYPs are involved in the metabolism for all three isomers (Table 1) it is obvious that the substitution pattern of the molecules govern their rate of metabolism, affecting the affinity of the enzyme for the individual TCBTs.

Furthermore, it was observed that, similar to the TCBTs, the more bulky PCBs were also metabolised by CYP2B, while the planar PCB 77 was metabolised by CYP1A1 and 1A2. In general, it appeared that more bulky molecules are preferably metabolised by CYP2B enzymes in both rat and humans (Lewis, 1997; Lewis *et al.*, 1999; Lewis and Lake, 1997), while planar PCBs and other planar molecules like polycyclic aromatic hydrocarbons *e.g.* benzo[a]pyrene were preferably metabolised by either CYP1A1 or CYP1A2 (Gonzalez and Gelboin, 1994; Zhang *et al.*, 1997). Thus, the observation that the TCBTs were metabolised by CYP2B enzymes in stead of CYP1A2 suggests that the TCBTs adopt a reltively non-planar conformation. This could imply that the interaction with the Ah-receptor, which requires a planar conformation, is not likely. Thus the potency of these TCBTs to induce dioxin-like toxicity via this receptor is expected to be low.

Extrahepatic metabolism and first-pass metabolism

Strong correlations were observed between metabolism of the three TCBTs and the examined *ortho*-substituted PCBs and CYP2B enzyme activities, and weaker correlations with CYP3A enzyme activities in both human and rat liver microsomes (Chapter 3 and 4). CYP2B and CYP3A in the rat are the major enzymes, with CYP3A occurring in the highest concentrations. In humans however, CYP2B6 is only a minor hepatic enzyme (0.2 % of the total CYP concentration) (Shimada *et al.*, 1994), while CYP3A4 is a major hepatic enzyme (30-40 % of the total CYP concentration) (De Wildt *et al.*, 1999; Gonzalez, 1992). This implies that although the concentration of CYP2B6 enzyme is low, the metabolism might be efficient. Consequently, the weaker affinity of CYP3A for the chlorinated compounds results in less efficient metabolism.

CYP identification was performed by correlation analysis and the use of selective inhibitors (Chapter 3 and 4), to elucidate whether extrahepatic tissue could contribute to the metabolism of the three TCBTs and the selected PCBs. CYP2B and CYP3A are the most important enzymes in the intestines and the lung of the rat (De Waziers *et al.*, 1990). In humans the relative amounts of CYP3A4 and CYP2B6 expressed in the intestines are comparable to that in the liver; CYP3A4 occurs in high concentrations (30-40 % of the total CYP concentration) (De Waziers *et al.*, 1990; De Wildt *et al.*, 1999; Gervot *et al.*, 1999; Zhang *et al.*, 1999) and the CYP2B6 concentrations are low (Gervot *et al.*, 1999). Thus after oral uptake first-pass metabolism in the intestines could occur, reducing the oral bioavailability of the TCBTs and the *ortho*-substituted PCBs in both rat and humans. In Chapter 6 this has indirectly been demonstrated for the three TCBTs in the rat. The oral bioavailability of these TCBTs in rat was 6, 20 and 48 % for TCBT 94, 88, and 87, respectively.

The contribution of the intestines to the first-pass metabolism could be anticipated because the compounds entered the organism via the GI-tract (Chapter 6). The contribution of the lungs is less obvious. The lungs could contribute to the first-pass metabolism in the case that the TCBTs are transported via the lymphatic route. After the lymph has mixed with the venous blood, the venous blood with the TCBTs is pumped to the lungs first, before reaching the systemic circulation. Rat lungs have a 1.5 times higher expression of CYP2B than the rat liver (De Waziers *et al.*, 1990), which could imply presystemic metabolism, *i.e.* first-pass, of the TCBTs in the lungs resulting in a reduced tissue concentrations. Chapter 6 was not conclusive about uptake either via the lymphatic route or via the portal vein route. However, in general many highly lipophilic substances are transported via the lymphatic system to the systemic circulation (Charman *et al.*, 1997). Thus, transport via the lymph of these highly lipophilic TCBTs and PCBs cannot be excluded. This implies that the lungs could play an important role in the first-pass metabolism of the studied compounds but also during the passage of systemic blood through the lungs.

The toxicity of TCBTs has been investigated sparsely while that of TCBT metabolites has not been subject of a research programme. With respect to PCBs, hydroxylated PCBs have been associated with inhibition of vitamin A and thyroxin transport, and with estrogenic effects (Brouwer and VandenBerg, 1986; Kramer *et al.*, 1997; Morse *et al.*, 1996). Thus, the lower bioavailability of the TCBTs by intestinal first-pass elimination, does not necessarily imply a reduction of the toxic risk.

Elimination

In the rat, terminal half-lives of the TCBTs in the blood, were comparable to those of PCB 77, 101 and 118, ranging from 3 to 4 days, while the *in vitro* metabolic rate constants of the TCBTs were 2 to 50 times faster relative to the PCBs. In humans the elimination of the TCBTs was slower, as indicated by their estimated long terminal half-lives, ranging from 22 to 111 days (Chapter 5, 6, and 7).

Figure 1 illustrates the effect of metabolic rate constants on the terminal half-lives in the rat using the PB-PK model (Chapter 5). The terminal blood half-life is sensitive to changes in the metabolic rate constants when metabolism is relatively slow (K*met*<1). Increasing K*met* does not affect the terminal half life substantially, indicating that metabolism is not the only factor determining the half-life of the TCBTs. It appeared from the sensitivity analysis in chapter 5, that the perfusion rate of the adipose tissue and the liver play a significant role in the amount that was metabolised.

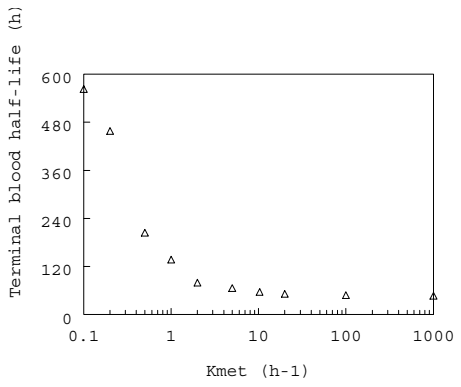


Figure 1. The effect of metabolic rate constants on the terminal half-life of TCBTs from the rat blood using the PB-PK model (Chapter 5)

Food chain accumulation

The kinetic studies with the TCBTs were performed to assess if TCBTs would accumulate throughout the food chain like PCBs. The major factor determining the differences in the toxicokinetics between the selected TCBTs and PCBs in mammals were the differences in metabolic rate constants. Since in fish, the metabolic rate constants of the TCBTs and the PCBs were slow and therefore hardly detectable (Chapter 2), it was assumed that the absorption, tissue distribution and elimination

would be analogous for both types of compounds like in mammals. This explains that the levels of the TCBTs and PCBs in fish lie in the same order of magnitude (Fuerst *et al*., 1987b; Leonards and DeVoogt, 1989; Wammes *et al*., 1997).

Due to the low oral bioavailability of the TCBTs in the rat, the tissue levels would be 52 to 95 % lower than for the PCBs at equal exposure levels. Similar low oral bioavailabilities were suggested for humans because of the presence of CYP2B6 and 3A4 in the human gut tissue. Furthermore, once absorbed the TCBTs are metabolised 2 to 50 times faster in the rat and 10 to 300 times faster in humans than the PCBs in the human liver. Based on our study we must conclude that food chain accumulation of TCBT to levels similar to PCBs is not likely to occur. This was confirmed by model simulation using a PB-PK model for humans (Chapter 7). The simulated tissue levels of TCBTs under steady state conditions were 100 to 1000 folds lower compared to measured human tissue levels of total PCBs and relatively low bioaccumulation factors (BAF) were calculated (eq. 1)

$$Bioaccumulation\ factor = \frac{Cf_{ss}}{F * Dose} \qquad \text{eq. 1}$$

where Cf_{ss} (μg/kg) is the steady state concentration in the adipose tissue of the TCBT or PCB and F is oral bioavailability and Dose is the daily dose in μg/kg.

For the TCBTs in humans BAF based on the estimated tissue levels, ranged from 0.03 to1.5, while for PCBs it ranged from 100 to 1000 using adipose tissue levels as reported in the literature. These calculations were also performed for TCBTs and PCBs in the rat. The BAF in the adipose tissue relative to the daily intake of 89 ng/kg/day was 4.8, 3.8 and 2.8 for TCBT 87, 88 and 94 respectively, while for PCB 77, 101 and 118 this was 9.2, 9.3 and 232, respectively. Thus, the accumulative potential of the TCBTs is a factor 2 to 100 lower than for the PCBs in the rat.

PB-PK MODELLING

The toxicokinetics were analysed with PB-PK models that were parameterised using physiology data from literature and *in vivo* tissue concentration data to derive partition coefficients (Chapter 5 and 6). The *in vivo* metabolic rate constants as well as the diffusional clearance constants were obtained by optimising the PB-PK model of the rat to the tissue concentration data.

This model was then scaled to the human situation (Chapter 7) to estimate the kinetics of TCBTs in humans. This scaling is associated with uncertainties with respect to the *in vitro-in vivo* extrapolation of human metabolism, and the interspecies extrapolation of the diffusional clearance constant. In the following paragraphs these uncertainties are being discussed.

In vitro-in vivo extrapolation

In vitro metabolic rate constants from the hepatic microsomes represent the metabolism in a subcellular fraction of the liver. Often these values can be extrapolated accurately to estimate metabolism *in vivo* (Carlile *et al.*, 1997; Hayes *et al.*, 1995; Houston, 1994b; Houston and Carlile, 1997). However, repeatedly, the estimates of the *in vitro* studies underestimate the *in vivo* metabolism (Obach, 1996). This was also demonstrated in chapter 5 for the three TCBTs. Insertion of the *in vitro* metabolic rate constant in the PB-PK model, underestimated the elimination from the rats with a factor 3 to 10.

In general, *in vitro* metabolic rates are obtained by incubating the substrate at a single concentration of microsomal protein in the incubation fluid. Chapter 7 illustrates that the metabolic rate constants are dependent on the amount of microsomal protein in the incubation fluid. Increasing the protein concentration increases the metabolic rate constant non-linearly. Since in the whole liver the concentration of the microsomal fraction is lower (0.045 mg/ml) than the commonly used 1 mg protein per ml, this suggests that extrapolation of one single *in vitro* metabolic rate constant would result in a good estimate of the *in vivo* metabolism by coincidence. This non-linear behaviour of the *in vitro* metabolism was explained by the free fraction of the substrate in the microsomes and in the incubation fluid as well as their respective volumes (VanEijkeren, 2000). Taking these factors into account provides estimates of the specific intrinsic clearance and the free fraction in the microsomes. Since the liver cells contain these microsomes exist as membrane bound enzymes in the hepatocyte, it is assumed that these parameter values are the same in the whole liver. However, partitioning in the whole liver is likely to deviate from that in the microsomes *e.g.* the lipid fraction is higher in the whole liver than in the microsomes in which more of the substrate could be dissolved, reducing the free fraction of the substrate. Now, the question arises whether the fraction that dissolves in the lipid fraction should be considered as bound or as still available for metabolism. This could be explored by increasing the lipid fraction in the incubation fluid and determining the effect on the metabolic rates.

The difference between the *in vitro* and *in vivo* estimates of the metabolism was suggested to be partly due to metabolism in the extrahepatic tissue. Especially the lungs could contribute significantly to the metabolism in the rat, as discussed previously, because of their high content of CYP2B relative to the liver (De Waziers *et al.*, 1990) which is one of the major enzymes metabolising the TCBTs (Chapter 3). In addition, hundred percent of the cardiac output flows through the lungs, while only 25 % of cardiac output perfuses the liver (Brown *et al.*, 1994). Thus, the lungs could metabolise significant amounts of the TCBTs. It is suggested that the *in vitro-in vivo* extrapolation would improve taking metabolism in this tissue into account. It appeared that the contribution of the intestines to the total metabolism after intravenous injection of the drug midazolam, which is metabolised by CYP3A in humans, was only 8%, while after oral administration this was 43%. This indicates that once in the systemic circulation the contribution of the intestines to the metabolism of midazolam becomes less important (Hall *et al.*, 1999). This suggests for the TCBTs that the contribution of the intestines to the metabolism has already been taken into account in the factor for the oral bioavailability which is low because of first-pass metabolism in the gut wall (Chapter 6). Thus, the lungs are one of the most likely organs that might explain the observed difference between *in vitro* and *in vivo* metabolic rate constants.

Diffusional clearance

The PB-PK model for TCBTs and PCBs in the rat includes a diffusional clearance constant to correct for the delay in tissue uptake by the adipose tissue, muscle and skin (Chapter 5 and 6). The metabolic rate constants and the diffusional clearance constants were obtained by optimising the PB-PK model to the tissue concentration data in rats. This PB-PK model was extrapolated to the human situation (Chapter 7). The diffusional clearance constants for the adipose tissue, skin, and muscle were inserted in the human model assuming that the uptake process in human tissue would be equal to that in rats. To assess the validity of this interspecies extrapolation, physiological insight in this constant is needed. Two possible uptake mechanisms are proposed here, that possibly provide a physiological interpretation of the diffusional clearance constant.

The diffusion limited uptake could be explained by the volume of the cells and the slower rate of perfusion. The tissue next to the artery or arteriole is in equilibrium with the blood, but deeper in the tissue this equilibrium does not exist yet, *i.e.* a large volume does not contain the compound. The diffusion of the compound from the

cell membrane to the centre is considered rate limiting (Lutz *et al.*, 1980). Thus, analysing the tissue when not all the tissue is in equilibrium with the blood will result in a lower tissue concentration. In the model the diffusional clearance constant is used to correct for this delayed uptake. However, determinants influencing this factor have not been studied. Assuming the diffusion limitation is explained by the volumes, then the diffusional clearance is expected to depend on the tissue it self, and not on the isomers. However, optimised parameter values of this constant for the TCBTs appear to depend on the isomer, while that of the PCBs appear to independent of the congener but dependent on the tissue.

The delayed uptake in these tissues could also be due to an active process that is rate limiting the uptake. It has been observed that lipoproteins, including chylomicrons, low densitiy lipoprotein (LDL), high density lipoprotein (HDL) and very low density lipoprotein (VLDL) play an important role in the transport of PCBs (Busbee *et al.*, 1985; Mohammed *et al.*, 1990; Noren *et al.*, 1999; Spindler Vomachka *et al.*, 1984). The PCBs equilibrate instantaneously between the lipoprotein fractions (Maliwal and Guthrie, 1982; Mohammed *et al.*, 1990) suggesting that the interactions are non-specific between the different lipoprotein fractions. The distribution of the PCBs depends solely on the concentrations of the different lipoprotein fractions (Matthews *et al.*, 1984). After absorption from the GI-tract the PCBs are transported via chylomicrons to the liver where they are processed (Borlakoglu *et al.*, 1990b; Busbee *et al.*, 1985).

Apart from the liver, the adipose tissue and the muscle contain receptors for the uptake of processed lipoproteins, including chylomicron remnants (Cooper, 1992), These receptors are known as lipoprotein lipases (LPL) and are both receptor and enzyme. LPL degrades the triglycerides to take up the free fatty acids. It is now hypothesised that the lipophilic chemicals are released because of the degradation of the lipoprotein, followed by tissue uptake. This process is assumed to be affected by the number of these receptors in the tissues.

The lipophilic vitamin A is dissolved in the lipoproteins and the chylomicron remnants that are recognised by the LPL receptor on the adipose tissue and the muscle. In mice overexpressing LPL resulted in higher tissue concentration of vitamin A, relative to mice with normal LPL expression (VanBennekum *et al.*, 1999). In addition, the adipose tissue and the muscle can take up whole lipoproteins (Karpe *et al.*, 1997). This suggests that PCBs or TCBTs could be taken up accordingly. This

receptor-mediated uptake via LPL has also been suggested by Borlakoglu *et al.* (1990b), however, data to support this hypothesis are still lacking. The skin also demonstrated a delayed uptake, but it does not have the lipoprotein receptors. However, the skin contains large adipose tissue depots subcutaneously, where the PCBs could be stored according to the process as described above. Thus it is hypothesised that the diffusional clearance constant depends on the number of LPL.

Human PB-PK model

The aim of this thesis was to compare the kinetics of three TCBTs with that of three PCBs in a quantitative way using PB-PK modelling and to estimate the possible accumulation of these compounds in humans.

The PB-PK models of these TCBTs and PCBs in the rat described the kinetics of both categories of compounds adequately (Chapter 5 and 6). The advantage of these models is that they can be extrapolated to other species by scaling the physiological parameters, biochemical and physicochemical parameters (Chapter 7), which entail the above-discussed uncertainties. Despite these uncertainties all information about the toxicokinetics of the TCBTs so far, including metabolism, bioavailability, and tissue to blood partitioning, have been integrated in the PB-PK model resulting in substantiated estimates of tissue concentrations in the male human. These estimates of the TCBTs were approximately 100 to 1000 fold lower (Chapter 7) than the PCBs levels, indicating the much lower potential of the TCBTs to accumulate in human tissue. Thus, the PB-PK model provided insight in the human kinetics of the highly lipophilic substances.

CONCLUSIONS

- Food chain accumulation of the TCBTs in mammals is up to two orders of magnitude lower in the rat and between two to three orders of magnitude lower in humans relative to the selected PCBs because of the faster metabolism of the TCBTs relative to the PCBs. In fish with their lower metabolic capacity accumulation of TCBTs occurs.
- The oral bioavailability of the TCBTs is lower than that of PCB 77, 101 and 118, which is due to high first-pass elimination of the TCBTs that most likely occurs in the small intestines rather than in the liver.
- CYP2B and CYP3A play an important role in the metabolism of the selected TCBTs and the non-planar PCBs, while the planar PCBs are metabolised by CYP1A enzymes. These enzymes are significantly expressed in the liver, the small intestines and the lungs. Thus these tissues can also contribute to the overall elimination of the TCBTs and the PCBs.
- *In vitro* metabolic rates of the TCBTs were only a factor 2 to 3 lower compared to the *in vivo* metabolic rates, indicating their use for parameterisation of PB-PK models.
- The *in vivo* kinetics of the three TCBTs equals that of the selected PCBs with respect to tissue distribution and disposition and, are evenly well described by the same PB-PK model representation. The individual parameter values reflected the compound specific properties.

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APPENDIX A

Mass balance differential equations

For adipose tissue, skin and muscle the amount in the tissue (Ax) and tissue blood (Axb) are described by the following differential equations where CA denotes the blood concentration, Qx, Cvx, PAx, Cx and Px denote the blood flow, venous blood concentration, diffusional clearance, and tissue: blood partition coefficient, respectively, associated with tissue x:

$$\frac{dAx}{dt} = PAx(Cvx - \frac{Cx}{Px}) \quad \text{eq.1}$$

$$\frac{dAxb}{dt} = Qx(CA - Cvx) + PAx(\frac{Cx}{Px} - Cvx) \quad \text{eq.2}$$

The amount in kidney and remaining tissue are described as follows:

$$\frac{dAx}{dt} = Qx(CA - \frac{Cx}{Px}) \quad \text{eq.3}$$

Concentrations in these tissues are represented by the amount divided by the tissue volume:

$$Cx = \frac{Ax}{Vx} \quad \text{eq.4}$$

Metabolism is described as a first-order process, which occurs in the liver. The liver compartment is therefore described as:

$$\frac{dAl}{dt} = Ql(CA - \frac{Cl}{Pl}) - Kmet * Al \quad \text{eq.5}$$

where K*met* represents the metabolic rate constant.

$$PAx = Qx * PAxC \quad \text{eq. 6}$$

Where Qx is the blood flow through tissue x and PAxC is a constant representing part of the diffusional clearance (PAx)

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SAMENVATTING

Technische mengsels van vetoplosbare polychloorbifenylen (PCBs) zijn gebruikt als vloeistof in transformatoren, condensatoren en als brandvertragers, vanwege hun thermostabiliteit, chemische stabiliteit en hun goede geleidbaarheid. Door vuilstort, lekkage uit industriële toepassingen en ongelukken zijn PCBs in het milieu terecht gekomen, waar ze persisteren door hun chemische stabiliteit. Vanwege het vetoplosbare karakter van de verbindingen kunnen de PCBs accumuleren in de voedselketen. Dit wil zeggen hoe hoger in de voedselketen, hoe hoger de geaccumuleerde concentratie.

PCBs blijken in diverse diersoorten toxische effecten te veroorzaken, zoals acute sterfte, leververgroting, verlies van lichaamsgewicht, atrofie van de thymus, onderdrukking van het immuunsysteem, reproductie- en ontwikkelingseffecten. De chemische stabiliteit, de accumulatie en de toxiciteit van de PCBs hebben ertoe geleid dat het gebruik ervan, mondiaal is verboden. Dit verbod leidde er vervolgens toe dat alternatieven werden ontwikkeld.

Ugilec 141 is een van die alternatieven. Dit technische mengsel bestaat uit tetrachlorobenzyltoluenen (TCBTs). Deze isomeren hebben vergelijkbare fysisch-chemische eigenschappen als PCBs. Dit deed vermoeden dat de TCBTs ook vergelijkbare problemen als PCBs zouden kunnen opleveren wat betreft persistentie in het milieu, en accumulatie in de voedselketen. Enkele toxiciteitstudies toonden aan dat de toxische effecten van TCBTs overeenkwam met die van PCBs, maar dat de effecten pas optraden bij hoger concentraties. Vanwege de verwachte problemen is in 1994 door de Europese Unie ook het gebruik van TCBTs in nieuwe apparatuur verboden.

In dit proefschrift is onderzocht of TCBTs in de voedselketen kunnen accumuleren en met name of de mens een risico loopt dit soort verbindingen te kunnen ophopen. Om deze vraag op te helderen is inzicht in de toxicokinetiek nodig. Dit houdt in alle processen die te maken hebben met opname van stoffen in het lichaam, verdeling van de stof over de verschillende weefsels en organen, afbraak van de stof (=metabolisme), en de uitscheiding. De kinetische processen kunnen gemodelleerd worden met behulp van op de fysiologie gebaseerde farmacokinetische modellen (PB-PK modellen). Deze modellen integreren fysiologische gegevens zoals bv. bloedstroomsnelheid, weefselvolume, en orgaangrootte, fysisch-chemische eigenschappen van de desbetreffende stof zoals bv. vetoplosbaarheid en

biochemische eigenschappen zoals bv. metabolisme snelheid. Het voordeel van dit soort PB-PK modellen is dat modellen die bijvoorbeeld voor de rat zijn ontwikkeld, vertaald kunnen worden naar de mens door fysiologische gegevens aan te passen aan die van de mens. Voor de ontwikkeling van dit soort modellen wordt vaak gebruik gemaakt van *in vitro* methoden om metabolisme snelheden te bepalen. Een van de moeilijkheden door het gebruik van *in vitro* methoden is dat de resultaten vertaald moeten worden naar *in vivo* waarden. Die extrapolatie brengt vaak een aantal onzekerheden met zich mee.

Van PCBs is bekend dat metabolisme de snelheidsbepalende stap is in de eliminatie van deze verbindingen. Dit betekent dat eenmaal gemetaboliseerd de PCBs snel het lichaam verlaten. Aangezien TCBTs lijken op PCBs is verondersteld dat dit ook voor hen geldt. Door te meten hoe snel de TCBT ten opzichte van de PCBs worden afgebroken kan een inschatting gemaakt worden of TCBTs meer of minder zullen accumuleren dan PCBs. Daarnaast, door de afbraaksnelheid te meten in verschillende diersoorten, kan tevens een indruk verkregen worden van de verschillen in accumulatie tussen diersoorten.

Dioxine en PCBs met een platte structuur kunnen interacties aangaan met de arylhydrocarbon (*Ah*) receptor, wat ten grondslag ligt aan hun specifieke toxiciteit. Om een interactie met de *Ah*-receptor aan te gaan is de grootte en de ruimtelijk structuur van het molecuul van belang. Moleculen met een platte (=planaire) conformatie zoals dioxines en planaire PCBs passen op die receptor. Ugilec 141 bestaat uit 69 isomeren. Hiervan zijn slechts drie onderzocht (nr. TCBT 87, 88 en 94), omdat theoretisch gezien deze drie isomeren een planaire conformatie kunnen aannemen en daardoor mogelijke een interactie kunnen aangaan met de *Ah*-receptor. Op basis hiervan zouden deze drie isomeren toxicologisch het meest relevant zijn.

In hoofdstuk twee is een methode beschreven waarmee de eerste en langzaamste stap in het metabolisme wordt gemeten. Die eerste stap is een hydroxylatie die door cytochrome P450 enzymen (CYP) wordt gekatalyseerd. Cytochrome P450 is een enzym systeem dat bestaat uit veel iso-enzymen. Die enzymen bevinden zich in zeer hoge concentratie in de lever en in lagere concentraties in andere organen en weefsels. De enzymen kunnen geïsoleerd worden door de lever fijn te malen en te centrifugeren. Het product dat uiteindelijk overblijft heet microsomen. Met deze microsomen van de ratte- en visselever zijn experimenten uitgevoerd om de afbraaksnelheid van de drie TCBTs te bepalen. De rattelever bleek de drie TCBTs

sneller af te breken dan de visselever. Ook bleken de CYPs met een verschillende snelheid de TCBTs af te breken: TCBT 94 >>TCBT 88>TCBT 87.

In hoofdstuk drie en vier is, met de methode uit hoofdstuk twee, de metabole afbraaksnelheid van de drie TCBTs en enkele PCBs bepaald in de lever van de rat en de mens. Bovendien is in die hoofdstukken ook beschreven welke CYPs de afbraak van die verbindingen bewerkstelligen. Uit beide hoofdstukken bleek dat zowel de levers van de mens en de rat TCBTs sneller afbreken dan PCBs. De gemiddelde afbraaksnelheid van TCBTs door de mens was vergelijkbaar met die van de rat. Deze resultaten zouden kunnen betekenen dat PCBs makkelijker accumuleren dan TCBTs in beide zoogdieren. De volgende iso-enzymen bleken betrokken te zijn bij de afbraak van de drie TCBTs (nr. 87, 88, 94) in de rat: CYP2B1, CYP2B2, CYP3A1,CYP3A2. In de mens waren dat de gerelateerde enzymen: CYP2B6 en CYP3A4. De PCB met een platte structuur (PCB 77) werd afgebroken door CYP1A1 en CYP1A2 in zowel de rat als de mens. De bolvormige PCBs (nr. 52, 101, 136) werden afgebroken door dezelfde enzymen als de TCBTs. Hieruit is geconcludeerd dat de TCBTs mogelijk bolvormig zijn, waardoor interactie met de *Ah*-receptor niet waarschijnlijk is. Dit impliceert dat ervan TCBTs geen dioxine-achtige toxiciteit te verwachten is.

In hoofdstuk 5 is de ontwikkeling van een PB-PK model voor TCBTs in de rat beschreven. Bovendien is in dit hoofdstuk een vergelijking gemaakt tussen de *in vitro* metabole afbraaksnelheid en de geschatte *in vivo* metabole afbraaksnelheid. Ratten zijn eenmalig intraveneus gedoseerd met een mengsel van de drie TCBTs. Op verschillende tijdstippen is gemeten in bloed, lever, spier, vet en de nier hoe hoog de concentratie van de TCBTs was. De fysiologische gegevens voor het model waren afkomstig uit de literatuur, de weefselverdeling van de TCBTs werd berekend uit de beschikbare weefsel-concentratie data. De waardes voor metabole afbraaksnelheid en de diffusiesnelheid van bloed naar huid, vet en spier werden geschat met behulp van het PB-PK model, door parameter waardes zo te variëren dat de modelvoorspelling zo goed mogelijk de geobserveerde data voorspelt. De voorspelde *in vivo* metabole afbraaksnelheid bleek slechts twee tot drie maal hoger te zijn dan de *in vitro* gemeten metabole afbraaksnelheid. Hieruit werd geconcludeerd dat de *in vitro* methode waardevolle gegevens opleverde voor het gebruik in PB-PK modellen.

In het algemeen worden PCBs en TCBTs via voedsel opgenomen. Die orale route kan de hoeveelheid van de verbindingen die het bloed bereikt (=biobeschikbaarheid) beïnvloeden. In hoofdstuk zes is de biobeschikbaarheid van

de drie TCBTs en enkele PCBs bepaald. Ratten kregen een mengsel van TCBTs en PCBs toegediend op een korrel voer. Op verschillende tijdstippen na consumptie is de concentratie van de TCBTs en de PCBs bepaald in bloed en weefsels. Bovendien is een mengsel van PCBs intraveneus toegediend. Met behulp van het PB-PK model uit hoofdstuk vijf en met een nieuw PB-PK model voor de PCBs na intraveneuze toediening, zijn de resultaten van de orale studie beschreven. Hiervoor is alleen de biobeschikbaarheid als parameter ingevoerd. De biobeschikbaarheid van TCBT 94 was 5%, TCBT 88 was 20% en TCBT 87 was 48% ten opzichte van de intraveneuze toediening. Voor de PCB 77, 101 en 118 was de biobeschikbaarheid respectievelijk: 60, 75, en 100%. De relatief lage biobeschikbaarheid van de TCBTs kon verklaard worden door afbraak van de verbindingen in het maagdarmkanaal, die relatief hoge concentraties bevatten van de enzymen die betrokken zijn bij de afbraak van de TCBTs.

In hoofdstuk zeven is een PB-PK model beschreven voor de kinetiek van TCBTs in de mens, wat de resultaten uit de bovenstaande hoofdstukken integreert. In hoofsdtuk vijf werd reeds aangetoond dat de *in vitro* metabole afbraaksnelheid goede resultaten opleverde om *in vivo* metabolisme te voorspellen in de rat. In hoofdstuk zeven is deze *in vitro-in vivo* extrapolatie nader onderzocht. Het PB-PK model voor de mens is gebruikt om weefselconcentraties te voorspellen na een eenmalige dosering en na 300 dagelijkse doseringen. Omdat er geen gegevens waren over weefselconcentraties van TCBTs in de dagelijkse voeding van de mens is een gemiddelde dagelijkse inname van PCBs gebruikt. De resulterende modelvoorspellingen zijn vergeleken met weefselconcentraties van PCBs die gemeten zijn in de mens. Er bleek dat de mens TCBTs 2 tot 100 keer minder accumuleert vergeleken met PCBs. Dit verschil kon o.a. verklaard worden door verschil in metabole afbraaksnelheid tussen TCBTs en PCBs

Uit de resultaten die beschreven zijn in dit proefschrift blijkt dat de lever microsomen van de forel niet in staat zijn de drie TCBTs meetbaar te metaboliseren, terwijl lever microsomen van de rat en de mens hiertoe wel instaat zijn. Hieruit is geconcludeerd dat vissen TCBTs wel accumuleren, maar dat zoogdieren, zoals bijvoorbeeld de mens, dit veel minder doen.

Bovendien is gebleken dat TCBTs sneller worden afgebroken dan PCBs door zowel de rat als de mens door met name CYP2B en CYP3A. Deze enzymen komen

tevens voor in andere weefsels dan in de lever, zoals de darm. Dit verklaart de geringe biobeschikbaarheid van TCBTs.

Door de snellere afbraak van TCBTs ten opzichte van PCBs en de geringe biobeschikbaarheid, zullen TCBTs slechts in geringe mate accumuleren bij een continue blootstelling. Tevens kan afgeleid worden uit de enzymen die betrokken zijn bij de afbraak van de TCBTs, dat *Ah*-receptor gemedieerde toxiciteit van TCBTs niet waarschijnlijk is. Daarmee zijn voor zoogdieren, inclusief de mens, de risico's, die verbonden zijn aan blootstelling aan TCBTs, te verwaarlozen. Dit in tegenstelling tot de aquatische organismen zoals vissen, die door trage afbraak TCBTs wel kunnen accumuleren.

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CURRICULUM VITAE

Hester Kramer is geboren te Oss op 14 december 1968. In 1987 behaalde zij haar VWO diploma aan het Maaslandcollege te Oss. In datzelfde jaar begon zij haar studie levenmiddelentechnologie aan de Landbouwuniversiteit te Wageningen. Zij verrichtte afstudeeronderzoek bij de vakgroep Toxicologie onder supervisie van Prof. Dr J. Koeman in het onderzoeksprogramma PCBs en moedermelk. Tevens verrichtte zij in haar afstudeerfase een epidemiologische studie bij de vakgroep Gezondheidsleer. In het kader van een europees uitwisselingsprogramma (ERASMUS) heeft zij 8 maanden gestudeerd aan de universiteit van Perpignan, Frankrijk. In 1993 behaalde zij haar doctoraalexamen levensmiddelentechnologie.

In september 1993 trad zij in dienst als projectmedewerker integrale normstelling bij het laboratorium voor Toxicologie, (later het laboratorium voor Effecten Onderzoek) van het Rijksinstituur voor Volksgezondheid en Milieu (RIVM) te Bilthoven. Zij verrichttet samen met Dr M.N. Pieters en Prof. Dr W. Slob onderzoek op het gebied van kwantitatieve humane risicoschatting. Dit onderzoek resulteerde in een aantal publicaties over het schatten van een veilige dosis met behulp van een beperkte set aan toxiciteitsgegevens.

Na drie jaar startte zij haar promotieonderzoek dat geïnitieerd werd vanuit UTOX, een samenwerkingsverband tussen de universiteit Utrecht, TNO-Voeding en het RIVM. Het onderzoek naar de farmacokinetiek van een PCB-vervanger werd met name uitgevoerd bij het Research Instituut Toxicologie (RITOX) van de Universiteit Utrecht en deels bij laboratorium voor blootstellingsonderzoek en milieuepidemiologie (LBM) van het RIVM. Tevens volgde zij naast haar promotieonderzoek, de postdoctorale opleiding Toxicologie.

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